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Award Number: W81XWH-05-2-0014

TITLE: Antingens for a Vaccine that Prevents Severe Malaria

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REPORT DATE: March 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2008	Annual	1 FEB 2007 - 31 JAN 2008

4. TITLE AND SUBTITLE

Antigens for a Vaccine that Prevents Severe Malaria

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-2-0014

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Seattle Biomedical Research Institute
Seattle, WA 98109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Malaria is the primary infectious disease threat facing the U.S. soldier, and is the leading cause of all causalities during tropical deployments. The long-term objective of this project is to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project will then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malaria anemia.

15. SUBJECT TERMS
Severe malaria, *P. falciparum,* microarrays, proteomic, vaccines

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	97	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION: Malaria is the primary infectious disease threat facing the U.S. solider, and is the leading cause of all casualties during tropical deployments. The long-term objective of this project is to identify and prepare the malaria parasite forms causing severe anemia, and then apply functional genomics and bioinformatics tools to identify 15 to 30 proteins that could form the basis for an effective vaccine at both the pre-erythrocytic and blood stages of malaria infection. The project will then evaluate these lead candidates for their recognition by sera collected from immune individuals, in order to identify the leading 3 to 5 candidates for a blood stage vaccine that prevents severe malarial anemia.

BODY: We have made good progress in many activities during our third year as described below.

Human Subjects Research Protocol

Our DOD protocol titled, "Antigens for a Vaccine that Prevents Severe Malaria" describes the functional genomics and immunoreactivity studies that we are performing on malaria parasites. After extensive revisions during the first grant year, it now supports laboratory work being conducted only at the Seattle Biomedical Research Institute in the U.S., and includes sample processing, immunoparasitology, and functional genomics studies on samples collected under a separately IRB-approved longitudinal cohort study. The longitudinal cohort study has previously been supported by funds from the National Institutes of Health and the Bill & Melinda Gates Foundation and is currently supported by a Grand Challenges grant from the Foundation for the National Institutes of Health.

We submitted the DOD protocol for continuing review to our local IRB (Western IRB in Olympia, WA) in December and received continuing approval on December 24, 2007 (annual approval expires January 6, 2009).

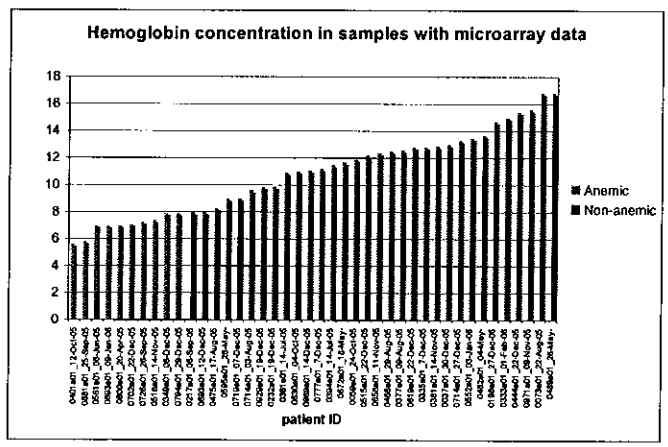
Optimization of Laboratory Methodology

Microarray Printing We have improved the quality of our oligonucleotide spotted microarrays by optimizing humidity and pin strike speeds as well as adjusting oligonucleotide concentrations. We have implemented quality control analyses to ensure quality and consistency of slides between different prints using open source (Bioconductor) and commercial (Acuity) programs.

RNA Extraction and Processing RNA extraction and purification from whole blood samples collected in Tanzania was improved and optimized. The RNA extraction time was reduced from 12 hours to 90 minutes and a procedure was added to remove globin mRNAs produced by reticulocytes, which compromises microarray sensitivity.

DNA Microarray Studies of Severe Anemia Parasites

We have processed the majority of peripheral blood samples from the Muheza cohort for microarray analysis. A total of 105 hybridizations performed with 42 samples from 40 individuals passed quality control filters. Ten of these samples are from patients with anemia (defined as blood hemoglobin concentration lower than 8 g/dL), and 32 are from patients who were classified as non-anemic (see Figure to right).



Using different bioinformatics tools to explore the resulting data, including Acuity, SAM, LIMMA and GenePattern, we compiled a list of approximately 75 candidate genes that appear to be up-regulated in parasite samples from anemic patients as compared to non-anemic patients. Interestingly, 6 of the 75 proteins in our candidate list (MAL7P1.174, PFD1170c, PFF0075c, PFE1605w, PF110037 and PFB0085c) belong to a family of predicted exported proteins of unknown function (PHISTb), which includes the vaccine candidate RESA. This family is conserved in other *Plasmodium* species but has particularly expanded in *P. falciparum* (Sargeant et al., Genome Biology 2006), and some PHISTb genes appear upregulated in parasites directly extracted from infected patients compared to the culture-adapted 3D7 (Daily et al., J.Inf. Dis. 2005).

We selected an initial subset of proteins that were identified by multiple analyses tools as being upregulated in anemic patients and have the characteristics of putative antigens for further analysis. These proteins have predicted signal peptide (SP) or transmembrane (TM) domains, signatures of exported proteins (Exp), and/or have been found in the membrane of the infected red blood cell by mass spectrometry (MS iRBCm), see Table below:

Candidate antigens		Known properties of candidates				Status			
		SP	TM	Exp	MS iRBCm	Cloned	Expressed	Ab raised	Bioplex
MAL7P1.174	PHIST b protein		1	+	+	√	√	√	√
PFD1170c	PHIST b protein			+		√			
PFF0075c	PHIST b protein			+?		√			
PFD1120c	etramp4	1	1			√	√	√	
PFB0095c	PfEMP3			+		√	√	√	
PFF0365c	putative signal transductor		10						

The differential expression of the mRNA corresponding to these proteins between anemic and non-anemic patients is currently being confirmed by quantitative PCR. In addition, several of these proteins are currently in our pipeline for cloning and cell-free protein expression. Successfully expressed candidates are being screened in Bioplex-based assays to determine whether sera from immune patients contain reactive antibodies against them, and antibodies against these proteins generated in mice are being used in flow cytometry experiments to test for reactivity against red blood cells infected with different parasite lines. The antibodies will also be used in immunofluorescence assays to determine the location of the candidates in the infected erythrocyte.

We have also conducted an experiment to validate the results obtained from our in-house spotted arrays using tiling arrays from Nimblegen. An initial comparison of the results from both platforms reveals a good correspondence between the most differentially expressed genes among samples. Because we only tested four patient samples in the tiling array platform, this test lacks the statistical power to allow us to identify genes up-regulated in anemia, however, two of the genes in our current short candidate list, MAL7P1.174 and PFF0365c, are found in the 95th and 90th percentile, respectively, when genes are sorted by the fold change between anemic and non-anemic samples in both microarray platforms.

With the goal of identifying other candidate genes that can be further explored with the tools described above, bioinformatic analysis of the results is ongoing. We have recently incorporated a new bioinformatics tool to our analysis, the non-negative matrix factorization algorithm, which classifies samples into clusters of similar gene expression without *a priori* assignment to specific categories. This approach allows the identification of the discriminating factors among clusters,

and can be used to better identify which samples to compare. We are also conducting regression analysis on the data to model factors such as anemia, parasitemia, age of the patient, parity, pregnancy malaria, hospitalization records and history of malaria infections into our candidate gene selection strategy. Furthermore, the available data can be analyzed to identify genes whose expression associates with other malaria-related features; for example, such as hyperparasitemia: 24 of the RNA samples analyzed by microarray to date correspond to patients with hyperparasitemia (more than 2500 parasites per 200 white blood cells), while 18 patients had lower levels of parasitic loads.

In addition to the peripheral blood samples we have already processed, we have over 200 samples that were cultured in the laboratory for approximately 48 hours. Analysis of these samples will allow us to identify candidate protein-encoding genes expressed in later phases of the intraerythrocytic cycle that could be responsible for the ability of parasitized erythrocytes to sequester.

Proteomics Studies of Severe Anemia Parasites

Two experimental approaches were used to identify proteins that are upregulated in parasites collected from children with severe anemia. The first approach employed a semi-quantitative LC-MS/MS method; the second approach employed quantitative proteomics using FTICR-MS. Using these proteomics approaches, we analyzed the IE surface proteome of parasites collected from children with anemia, in comparison to that of parasites collected from children with other clinical syndromes like hyperparasitemia or of placental parasites.

In these quantitative proteomics studies, we found that 10 proteins were expressed at significantly higher levels in parasites from children with anemia. Fold change in mean protein abundance varied between 3.2-78 (see table below). Three of these proteins were also identified by semi-quantitative methods to be associated with malarial anemia. Five of the proteins contain predicted transmembrane domain (TM). To validate that these proteins are upregulated in parasites associated with anemia, we are in the process of producing these proteins in recombinant form, using both eukaryotic cell-free expression system and E. coli expression system.

Name	Gene ID	SP	TM	Fold change: anemia vs non anemia	Fold change: anemia vs placenta	*p* value
serine repeat antigen-6	PFB0335c		y	3.21596068	15	<.0001
HSP60	PFF0590c		y	15.6874945	8.457412	<.0001
translation initiation factor-like protein	PF08_0018			3.43509051	71.82496405	0.0005
hypothetical protein	MAL13P1.299	y	y	8.38567112	39.27753496	<.0001
RNA-binding protein	PFI1025w			24.3827058	49.98633087	<.0005
proliferation-associated protein	PF14_0261			6.15967005	6.012381281	0.0007
cAMP-dependent protein kinase	PFL1110c			26.5765229	8.225850112	0.007
hypothetical protein	PFC0760c		y	78.014611	36.82168487	0.0003
hypothetical protein	PF08_0137		y	24.1037728	64.37807161	<.0001

KEY RESEARCH ACCOMPLISHMENTS: Below is a list of key research accomplishments emanating from this research:

- Human Subjects Protocol Continuing Review Approvals obtained

- Identified several known surface protein genes (called *var* genes) that are upregulated in parasites causing anemia
- Identified several hypothetical protein genes that are upregulated in parasites from anemic patients that would also make good vaccine antigen candidates
- Established cell-free protein expression in our laboratory using ENDEXT technology to prepare recombinant proteins for serosurveys
- Continued to improve the quality of our oligonucleotide spotted microarrays and implemented quality control analyses to ensure quality and consistency of slides between different prints
- Continued to improve RNA stabilization and extraction assays
- Completed microarray studies of parasites causing severe anemia in Tanzania

REPORTABLE OUTCOMES:

Publications

1. Dickert N, DeRiemer KL, **Duffy PE**, Garcia-Garcia L, Mutabingwa TK, Sina B, Tindana P, and Lie R. 2007. Ancillary Care Responsibilities in Observational Research: Two Cases, Two Problems. **Lancet**, 369 (9564):874-7.
2. A Muehlenbachs*, TK Mutabingwa, M Fried, **Duffy PE**. 2007. An unusual presentation of placental malaria: a single persisting nidus of sequestered parasites. **Human Pathol**, 38(3):520-3. Epub 2007 Jan 19.
3. Muehlenbachs A*, TK Mutabingwa, M Fried, **Duffy PE**. 2007. Genome wide expression analysis of placental malaria reveals features of lymphoid neogenesis during chronic infection. **J Immunol**, 179:557-65.
4. Oleinikov AV, E Rossnagle, S Francis, TK Mutabingwa, M Fried, **Duffy PE**. 2007. Effects of sex, parity, and sequence variation on seroreactivity to candidate pregnancy malaria vaccine antigens. **J Infect Dis**, 196:155-64.
5. M Fried, KK Hixson, L Anderson, TK Mutabingwa, **Duffy PE**. 2007. The distinct surface proteome of placental malaria parasites. **Mol Biochem Parasitol**, 155:57-65.
6. Francis SE, Malkov VA, Oleinikov AV, Rossnagle E, Wendler JP*, Mutabingwa TK, Fried M, **Duffy PE**. 2007. Six genes are preferentially expressed by the circulating and sequestered forms of *Plasmodium falciparum* parasites that infect pregnant women. **Infect Immun**, 75:4838-50.
7. **Duffy PE**. 2007. *Plasmodium* in the placenta: parasites, parity, protection, prevention, and possibly pre-eclampsia. **Parasitol**, 134:1877-81.
8. Ntoumi F, Kwiatkowski DP, Diakite M, Mutabingwa TK, **Duffy PE**. 2007. New interventions for malaria: mining the human and parasite genomes. **Am J Trop Med Hyg**, 77:S270-S275.
9. Kabyemela ER*, Muehlenbachs A*, Fried M, Kurtis JD, Mutabingwa TK, **Duffy PE**. 2007. Maternal Peripheral Blood Level of IL-10 as a Marker for Inflammatory Placental Malaria. **Malaria J**, 7(1):26.
10. Oleinikov AV, Francis S, Dorfman JR, Rossnagle E, Balcaitis S, Getz T, Avril M, Gose S, Smith JD, Fried M, **Duffy PE**. 2007. VAR2CSA domains expressed in *E.coli* induce cross-reactive antibodies to native protein. **J Infect Dis**, in press.
11. Greenwood BM, Fidock DA, Kyle DE, Kappe SHI, Alonso P, Collins F, **Duffy PE**. 2007. Malaria: Progress, perils, and prospects for eradication. **J Clin Invest**, in press.
12. Kabyemela ER*, Fried M, Kurtis JD, Mutabingwa TK, **Duffy PE**. 2007. Fetal responses during placental malaria determine the risk of low birth weight. **Infect Immun**, in press.
13. Avril M, Kulasekara BR, Gose SO, Rowe C, Dahlbäck M, **Duffy PE,** Fried M, Salanti A, Misher L, Narum DL, Smith JD. 2007. Evidence for globally shared, cross-reacting polymorphic epitopes in the pregnancy malaria vaccine candidate VAR2CSA. **Infect Immun**, in press.

14. Harrington W*, **Duffy PE**. 2007. Congenital malaria: rare but potentially fatal. **Ped Health**, in revision.
15. Kabyemela ER*, Fried M, Kurtis JD, Mutabingwa TK, **Duffy PE**. 2007. Decreased susceptibility to *Plasmodium falciparum* in pregnant women with iron deficiency. **J Infect Dis**, in revision.
16. Kabyemela ER*, Fried M, Kurtis JD, Gwamaka M*, Muehlenbachs A*, Mutabingwa TK, **Duffy PE**. 2007. Fetal anemia in Tanzania is related to α^{+}-thalassemia and inflammatory cytokine balance. Submitted.
17. Muehlenbachs A*, TK Mutabingwa, J Lachowitzer, M Fried, **Duffy PE**. 2007. FLT1 is a malaria resistance gene under natural selection *in utero*. Submitted.

Presentations

1. Seminar speaker. "Malaria during Pregnancy: Epidemiology, pathogenesis, immunology." Statistical Center for HIV/AIDS Research and Prevention, Fred Hutchinson Cancer Research Center. 11 September 2006.
2. Symposium Speaker. "Field studies in Africa." GCGH All Grantee Meeting, Washington, D.C. 4-6 October 2006.
3. Symposium Speaker. "Parasite diversity and the spectrum of disease due to P. falciparum." ASTMH 55th Annual Meeting, Atlanta, Georgia, 12-16 November, 2006.
4. Invited Speaker. "Pathological effects of pregnancy malaria on mother and offspring." Israeli Society for Parasitology, Protozoology, and Tropical Diseases Annual Meeting and Severe Malaria Workshop, Maale Hahamisha, Israel. 13-14 December 2006.
5. Symposium Speaker. "Malaria in Pregnancy." Shoklo Malaria Research Unit 20 Years Anniversary Seminar, Mae Sot, Thailand. 28 December 2006.
6. Symposium Speaker. "Functional genomics tools and field studies of *Plasmodium falciparum*." Malaria Parasite Diversity Meeting, Hinxton, Cambridgeshire. 20-22 January 2007.
7. Invited Speaker. "Malaria control strategies: The human immune system." Western Regional International Health Conference, Seattle, Washington. 16-18 February 2007.
8. Symposium Speaker. "Malaria Control." Whitehead Institute annual press conference, Cambridge, MA. 23 April 2007.
9. Seminar Speaker. "Malaria at the mother-child interface." Albert Einstein College of Medicine, Bronx, NY. 9 May 2007.
10. Co-organizer. Symposium: "The molecular background to severe and complicated malaria." Karolinska Institutet. Stockholm, Sweden. 14-16 June 2007.
11. Co-organizer. East African Regional Training Workshop: "Genomic Studies of Protozoan Pathogens." Sokoine University. Morogoro, Tanzania. 1-14 July 2007.
12. Symposium Speaker. "Malaria in Pregnancy." Gordon Conference, Oxford, UK. 9-14 September 2007.
13. Symposium Speaker. "Protective immunity to severe malaria in African children." Keystone Symposium, Challenges of Global Vaccine Development, Cape Town, South Africa. 8-12 October 2007.
14. Hematology Grand Rounds Speaker. "Malaria pathogenesis at the mother-child interface." University of Washington. Seattle, WA. 18 January 2008.
15. Symposium Speaker. "Fetal responses during placental malaria." MAM Conference. Melbourne, Australia. 3-7 February 2008.
16. Invited Speaker. Institute for Health Metrics & Evaluation. Seattle, WA. 13 March 2008.
17. Invited Speaker. Malaria Next Steps. London, UK. 8-9 April 2008.
18. Keynote Speaker. BioMalPar 4th annual meeting on the "Biology and Pathology of the Malaria Parasite." Heidelberg, Germany. 14-16 April 2008.

19. Symposium Speaker. Keystone Meeting, Malaria: Immunology, Pathogenesis and Vaccine Perspectives. Alpbach, Austria. 8-13 June 2008.

CONCLUSION: We have completed the microarray studies of parasites causing severe anemia in Tanzania. Theses studies have identified several parasite genes that appear to be preferentially transcribed by severe malaria parasites. These studies are now being confirmed using parasites causing severe anemia at another study site in Morogoro Tanzania. We will continue our seroepidemiology studies to examine whether antibody responses against these severe anemia parasite antigens correlate with resistance to severe anemia. Evidence that the antibodies correspond to protection will provide a strong rationale for further development of these antigens as vaccine candidates against malarial anemia.

REFERENCES: None.

APPENDICES: Publication numbers 1 through 9.

SUPPORTING DATA: None.

Ancillary-care responsibilities in observational research: two cases, two issues

N Dickert, K DeRiemer, P E Duffy, L Garcia-Garcia, T K Mutabingwa, B J Sina, P Tindana, R Lie

Lancet 2006; 368: 874–77

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International collaborative research in developing countries raises difficult ethical issues in the setting of severe diseases and complex costly treatments. Discussion of two matters has characterised the debate on this type of research. First, what standard of care should be provided to participants in intervention studies, particularly those in control groups?[1–4] Second, what level of benefits should be provided to individuals and communities during a study and after completion, particularly with respect to treatments proven effective through research?[4–6] Here, we focus on a third issue, investigators' responsibilities for meeting participants' needs for ancillary care.

Ancillary-care needs have been identified as distinct only in the past few years.[7,8] Richardson and Belsky demarcated such care as "that which is not required to make a study scientifically valid, to ensure a trial's safety, or to redress research injuries".[7,8] Examples include treatment of schistosomiasis detected in the urine of participants in a malaria study and management of HIV acquired in a preventive HIV vaccine trial.[7] Requirements for ancillary care can arise in any study in which people with unmet health needs are enrolled, but no policies or guidelines exist with respect to the researchers' duty to meet such needs. After briefly describing Richardson and Belsky's framework to set out ancillary-care responsibilities, we discuss two cases that raise frequently faced but important challenges, which we believe have not received sufficient attention in existing published work.

Richardson and Belsky propose a two-question framework to establish duties of ancillary care. First, does treatment for a particular health need fall within the so-called scope of entrustment between participant and investigator? Second, how strong is the individual's claim to that care? Richardson and Belsky argue that the investigator-participant relation is determined by the subset of health needs that participants entrust to the investigator when agreeing to take part in a clinical research study. Investigators' responsibility for provision of care is restricted to care addressing that subset of participants' health needs. Such needs are the ones for which researchers have some responsibility for provision of care. The scope of entrustment is thus "fixed by the subset of the permissions obtained during the consent process that are required for the research team to carry out the study validly and safely"[7] and typically includes caring for the disease under study and "following up on any clinically relevant information or diagnoses generated".[8] If a specific form of care is within the scope of entrustment, the participant does have some claim to that care. The strength of the individual's claim is then ascertained by the degree of the individual's vulnerability and dependence, the depth of the relation between investigator and participant, the extent of gratitude owed to the individual for accepting uncompensated burden, and the importance of reasons against providing care. Such reasons might include, for example, scientific considerations, cost, duration of treatment, or deficient infrastructure.[7,8]

Richardson and Belsky stress the need for ongoing dialogue to provide answers for these two questions in particular studies. The cases we discuss next, both funded by US sponsors, contribute to that process. The first case, however, also poses a challenge, suggesting that the range of ancillary-care responsibility is defined by more than entrustment of specific health needs and could be wider than Richardson and Belsky propose.

The first case, which illustrates the challenge of establishing the investigators' scope of responsibilities, is the Mother-Offspring Malaria Study (MOMS), a 5-year observational study focused on the pathogenesis and clinical outcomes of severe malarial disease in children. Participants were mother-infant pairs at the Muheza Designated District Hospital (MDDH) in Tanzania and were enrolled around the time of delivery.[9] The protocol included obtaining peripheral, placental, and umbilical-cord blood at delivery, capillary blood at 2-week intervals for the first year of life then every month until age 4 years, and venous blood every 6 months.

Children in MOMS received all care (including treatment for malaria) from MDDH, with all costs paid by the project. Additionally, a coinvestigator reviewed case records for enrolled children during the study to ensure that they were receiving appropriate inpatient and outpatient care. Despite the high prevalence of HIV in the local population, the hospital could not afford antiretroviral drugs and did not routinely provide prophylaxis for HIV-associated opportunistic infections. The annual government expenditure on health care was only US$12 per person.

During the first year of MOMS, several children with HIV died from opportunistic infections. Study investigators then developed a plan with the hospital to refer all children with persistent HIV-positive antibody status to a hospice clinic and to provide co-trimoxazole to all offspring of HIV-infected mothers. At least two types of ancillary care were thus provided: 1) monitoring to ensure proper malaria care; and 2) hospice referral for HIV-related treatment, including co-trimoxazole prophylaxis.

Monitoring of malaria treatment was clearly within the scope of entrustment as articulated by Richardson and Belsky. Malaria was the disease under study, and

participants probably expected investigators to take some responsibility for their malaria treatment, particularly because the study had the potential to generate clinically relevant information about their disease. A duty to provide care for HIV-related illness, however, seems to fall outside the scope of entrustment. Knowledge about HIV status is one of many elements of the medical history that were relevant to investigators, but data on HIV were not generated by participation, were not central to the research question, and were available to anyone interacting clinically with participants. Although HIV-related care was thus outside the scope of entrustment as defined by Richardson and Belsky, MOMS investigators felt a sense of duty to ensure hospice referral and prophylaxis for opportunistic infections for sick participants. Was this ancillary care supererogatory—that is, morally praiseworthy but optional—or is Richardson and Belsky's notion of the range of responsibility incomplete?

We believe that the study investigators recognised that their responsibilities to provide ancillary care extended beyond what was entrusted by individuals agreeing to participate. In particular, they recognised that the need for prophylaxis for opportunistic infections was important, that effective treatment was affordable, and that they had an ongoing research relation with participants. These factors play an important part in Richardson and Belsky's framework by helping to ascertain the strength of responsibilities within the scope of entrustment (ie, malaria-related treatment), but they cannot strengthen a duty that is outside this range (ie, HIV-related treatment). We see no reason why these factors ought not to have a role in establishing the scope as well.

Using Richardson and Belsky's framework, the way in which investigators planned to use information about HIV infection in the MOMS project is especially relevant to the task of assessing whether some responsibility exists. If adjustment for or stratification by HIV infection were part of the investigators' analytical plan, for example, HIV-related care might fall within the scope of entrustment. But, if HIV status were gathered only as part of an initial history, such treatment would not be included. Why does relevance of information to the aim of the study indicate the level of responsibility that participants entrust? Have participants disclosing HIV infection as part of the routine medical history taken at the beginning of most research projects really entrusted less than individuals whose HIV status will be central to data analysis?

We agree with Richardson and Belsky that understanding the nature of the investigator-participant relation is essential to locating ancillary-care responsibilities. Furthermore, we concur that such a relation exists in some middle ground between a clinical provider and a detached scientist and varies substantially based on the nature of specific studies. However, there is more to this relation than what is entrusted by people in agreeing to participate in a study. Moreover, what is entrusted seems to depend less heavily on the nature of the study than Richardson and Belsky suggest.

Some researchers might argue that the MOMS project presents a straightforward duty of assistance—or duty of rescue—and that the scope requirement is unhelpful. Singer's position, for example, suggests that to withhold treatments or services from research participants, which a study can provide and to which individuals would not otherwise have access, would be ethically unjustifiable.[10] Richardson and Belsky might even admit a duty of assistance in the MOMS case, asserting that entrustment responsibilities are distinct from duties of assistance and depend only on the nature of research and the investigator-participant relation. We are sympathetic to their desire to locate the responsibilities that inhere in the nature of the relation between investigators and patients, but we are concerned that entrustment provides an incomplete account of the relation. Our analysis thus calls the scope requirement into question and could suggest that what is needed is a deeper context-specific understanding of researchers' duties of assistance. The boundaries of the range of investigators' responsibilities

Tuberculosis case finding by community health workers in a rural household in southern Mexico

remain unclear. At the very least, however, we suggest that some criteria relevant to assessment of the strength of a claim are important determinants of the scope of researchers' specific duties.

This case is challenging with respect to scope, but the strength of participants' claims seems strong. The debt of gratitude is small; participants accept few uncompensated risks. However, they have no other source of care and are very vulnerable, and investigators are engaged deeply with the community and individuals. Finally, no strong reasons exist against providing this treatment; it was inexpensive and could be provided through the hospital. As in the second case, however, assessment of strength can, at times, be challenging.

The second case, which illustrates the challenges in determining the strength of participants' claims, is a population-based prospective study of the effect of short-course directly observed treatment (DOTS) on drug resistance and transmission of tuberculosis between 1995 and 2000 in southern Mexico.[11] In this observational study investigators partnered with the existing local DOTS effort and used passive case-finding by community health workers and conventional diagnostic and molecular epidemiological methods to detect study endpoints (figure). Initially, in the local DOTS programme, individuals with new infections were treated with three first-line anti-tuberculosis drugs; those who needed retreatment received an additional fourth agent. In 1998, local health authorities—with the encouragement of the study team and in accordance with WHO recommendations—began treating people with new infections with four drugs and retreatment cases with five agents. Neither the local health department nor the research study provided second-line drug treatment for multidrug-resistant disease; however, all participants were referred to the national tuberculosis control programme and were assessed by doctors of the National Institute of Respiratory Diseases, the national referral centre.

Over the 5-year study period, researchers noted that, even with moderate levels of multidrug-resistant tuberculosis, DOTS (with only first-line agents) rapidly reduced the incidence of drug-susceptible and drug-resistant infections. In particular, the regimen significantly lowered transmission of multidrug-resistant disease, although it did not decrease deaths in individuals resistant to more than one first-line drug or with multidrug-resistant tuberculosis. The strategy simply rendered these people non-infectious. In total, 41 participants were identified with multidrug-resistant tuberculosis; five died and only eight (19·5%) were judged a treatment success.

In view of the high mortality and failure rates in participants with multidrug-resistant tuberculosis, should investigators have treated these people with second-line anti-tuberculosis drugs? Individuals were treated with the local standard of care and in accordance with available international guidance; WHO did not recommend treatment of multidrug-resistant tuberculosis with second-line drugs at any point during the study. However, the standard of care in the USA at that time was to treat this disease with individualised regimens of second-line agents.[12] The cost for such treatment is nearly US$28000 per patient,[13] or $1148000 to treat 41 people, far more than the small budget of this observational study. Although, it does not refer explicitly to ancillary-care issues, the Declaration of Helsinki states clearly that the best treatment worldwide must always be provided to research participants, a position not affirmed definitively until October, 2000 (after this study was concluded). More importantly, this position is not shared widely. Other international guidance documents do not include the same requirement, and whether it is ethically defensible is not clear.[1]

Treatment of multidrug-resistant tuberculosis is arguably within the investigators' scope of responsibility. They were never entrusted with any health-care decision-making in this observational study. Richardson and Belsky[7] propose that the scope of entrustment depends primarily on the nature of the study and is fixed by the subset of the permissions obtained during the consent process. However, investigators had privileged access to individualised information about drug sensitivity, members of the research team were involved in optimisation of the DOTS programme, and investigators were involved in increasing access to treatment for people with susceptible and resistant tuberculosis, both locally and nationwide. The strength of participants' claims to second-line treatment of multidrug-resistant tuberculosis, however, is even less clear. The participants' vulnerability and dependence were indisputable since the illness is largely fatal and no other sources of treatment were available. However, individuals assumed no uncompensated risks, and investigators did not have a deep relation with participants; the research team was generally observing the existing DOTS effort. Most importantly, there were strong economic and scientific reasons against providing individualised treatment with second-line drugs. Treating participants at a cost of $1148000 was impossible on this study's budget. Additionally, the study was designed to show the effect of DOTS (with only first-line agents) on multidrug-resistant disease transmission; the finding that DOTS is effective in this respect has important implications for the design of tuberculosis treatment programmes. Had participants been treated with second-line agents, this effect could not have been detected.

Was this study ethically problematic as implemented? The Declaration of Helsinki suggests that it could be improved.[4] Furthermore, some commentators would challenge the legitimacy of economic reasons against providing treatment and the true scientific importance of knowing that first-line drugs reduced transmission of multidrug-resistant disease, asserting that both are only relevant as a result of injustice.[14] Important inequities are at the root of issues such as tuberculosis in developing

countries, but this matter does not invalidate, or make complicit, research aimed at improving health in the presence of such inequities. The Helsinki position is untenable if this important research is to proceed.

After conclusion of this DOTS study, a standardised regimen of second-line drugs was shown to be effective for treatment of multidrug-resistant tuberculosis (although less effective than individualised therapy), at a cost of around $2000 per patient.[15] The cost of treating 41 patients with the disease would then be $82 000 versus $1 148 000. The strength of participants' claims to treatment might thus increase. However, the need to reduce transmission, the high cost of treatment, and the low cure rate with all available treatment regimens would remain relevant to such assessments. Exactly how we weigh these competing factors remains a difficult challenge.

This second case indicates the tensions that exist between vulnerability, dependency, cost, and scientific considerations. It provides an especially helpful example of the need to prevent extreme vulnerability and dependence from hijacking all other considerations, an unfortunate outcome of the position advocated in the Declaration of Helsinki and by several authors in published work.[4,14] Such stances could entail the loss of valuable research findings for communities with important health needs. One great advantage of Richardson and Belsky's framework is that it explicitly requires that these important moral considerations be assessed in context; one consideration cannot trump all others.

In conclusion, the most important implication of this discussion has to do with the range of investigators' duties to provide ancillary care. The MOMS project challenges directly Richardson and Belsky's notion of the investigator-participant relation and the scope of ancillary-care responsibilities. We have suggested that the scope is established by more than what is entrusted to investigators by participants. We have also challenged the notion that the tightness of the connection between a particular ancillary-care need and the research question is an especially important determinant. The range of ancillary-care responsibility is not boundless, and we agree with Richardson and Belsky that it is mainly delimited by the nature of the research relation. However, factors such as the existence of need, the ability to help, and particularly investigators' level of engagement with participants are all important elements of that association and are thus material to establishing the scope of responsibility in addition to determining the strength of claims to care.

We also hope to have called attention to the many factors that determine the strength of a particular claim to ancillary care by research participants. The DOTS study indicates a serious flaw with the hard-line position that participants must be assured of the best treatment available anywhere. Requiring investigators to provide individualised second-line treatment would have made the study prohibitively expensive and prevented detection of important endpoints. Insistence on such a requirement inflates ancillary-care duties and fails to recognise the important role of research in advancing health in the developing world.

Ancillary-care needs are ubiquitous and warrant interest by the bioethics and research communities. Richardson and Belsky should be commended for recognising these issues as distinct and calling attention to their importance. We hope that discussion of these two cases will advance our understanding of ancillary-care responsibilities and facilitate ethical implementation of important research addressing health issues in developing countries.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We thank Rachel Derr, Ezekiel Emanuel, Christine Grady, Franklin Miller, Alfredo Ponce-de-Leon, Jose Sifuentes-Osornio, and Henry Richardson for helpful comments on earlier versions of this report; the audience at the 2004 annual meeting of the American Society for Tropical Medicine and Hygiene, at which these cases were discussed; and the Fogarty International Center for facilitating this collaboration.

References

1 Lie RK, Emanuel E, Grady C, Wendler D. The standard of care debate: the Declaration of Helsinki versus the international consensus opinion. *J Med Ethics* 2004; 30: 190–93.

2 Lurie P, Wolfe SM. Unethical trials of interventions to reduce perinatal transmission of the human immunodeficiency virus in developing countries. *N Engl J Med* 1997; 337: 853–56.

3 Varmus H, Satcher D. Ethical complexities of conducting research in developing countries. *N Engl J Med* 1997; 337: 1003–05.

4 WMA. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. Ferney-Voltaire: World Medical Association, 2000.

5 Participants in the 2001 Conference on Ethical Aspects of Research in Developing Countries. Moral standards for research in developing countries: from "reasonable availability" to "fair benefits". *Hastings Cent Rep* 2004; 34: 17–27.

6 Council for International Organizations of Medical Sciences. International ethical guidelines for biomedical research involving human subjects. Geneva: Council for International Organizations of Medical Sciences, 2002.

7 Richardson HS, Belsky L. The ancillary-care responsibilities of medical researchers: an ethical framework for thinking about the clinical care that researchers owe their subjects. *Hastings Cent Rep* 2004; 34: 25–33.

8 Belsky L, Richardson HS. Medical researchers' ancillary clinical care responsibilities. *BMJ* 2004; 328: 1494–96.

9 Mutabingwa TK, Bolla MC, Li JL, et al. Maternal malaria and gravidity interact to modify infant susceptibility to malaria. *PLoS Med* 2005; 2: e407.

10 Singer P. Famine, affluence, and morality. *Philosophy and Public Affairs* 1972; 1: 229–43. Available at: http://www.utilitarian.net/singer/by/1972----.htm (accessed Oct 20, 2006).

11 DeRiemer K, Garcia-Garcia L, Bobadilla-del-Valle M, et al. Does DOTS work in populations with drug-resistant tuberculosis? *Lancet* 2005; 365: 1239–45.

12 Goble M, Iseman MD, Madsen LA, Waite D, Ackerson L, Horsburgh CR Jr. Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. *N Engl J Med* 1993; 328: 527–32.

13 Burgos M, Gonzalez LC, Paz EA, et al. Treatment of multidrug-resistant tuberculosis in San Francisco: an outpatient-based approach. *Clin Infect Dis* 2005; 40: 968–75.

14 Schuklenk U. The standard of care debate: against the myth of an "international consensus opinion". *J Med Ethics* 2004; 30: 194–97.

15 Suarez PG, Floyd K, Portocarrero J, et al. Feasibility and cost-effectiveness of standardised second-line drug treatment for chronic tuberculosis patients: a national cohort study in Peru. *Lancet* 2002; 359: 1980–89.

Human Pathology (2007) 38, 520–523

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Case study

An unusual presentation of placental malaria: a single persisting nidus of sequestered parasites☆

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Received 26 July 2006; revised 15 September 2006; accepted 15 September 2006

Keywords:
Malaria-falciparum;
Pregnancy;
Placenta;
Histology

Summary Placental malaria caused by *Plasmodium falciparum* is a public health concern in tropical countries. Peripheral blood smears to detect placental malaria are often negative, and recrudescences are common during pregnancy. We performed placental histology on a series of first-time mothers delivering in an area endemic for *P falciparum*. A single nidus of malaria-infected erythrocytes was identified by placental histology in a single intervillous space from a woman who had no other evidence of peripheral or placental blood parasitemia. This finding suggests ring stage–infected erythrocytes sequester in vivo, or *P falciparum* can persist as a dormant blood stage form.

1. Background

The protozoan *Plasmodium falciparum* is 1 of 4 human malaria parasites that multiply within erythrocytes during their asexual blood-stage development. Acute, life-threatening complications of falciparum malaria are related to adhesion of mature-stage infected erythrocytes (IE) to endothelium, which allows IE to sequester in deep vascular beds of various organs. Immature ring stage IE circulate in the peripheral blood and are generally thought not to be adherent. The diagnosis of falciparum malaria often relies on microscopy to detect ring-stage parasites in peripheral blood smears stained with Giemsa, but histology and molecular techniques can also be used.

Pregnant women are susceptible to falciparum malaria, particularly during first pregnancies, and treatment failures are common. In a study from Kenya, recrudescences after treatment were 2- to 6-fold more common in women during first pregnancy than in nonpregnant women [1]. The pathogenesis of placental malaria (PM) involves the sequestration

No conflicts of interest reported. The views expressed in this article do not necessarily reflect those of the US Department of Defense.

☆ The Mother-Offspring Malaria Studies (MOMS) Project is supported by grants from US National Institutes of Health (R01 AI 52059 from National Institute of Allergy and Infectious Diseases [Bethesda, MD] and TW 05509 from Fogarty International Center) and the Bill and Melinda Gates Foundation (Seattle, WA). A.M. is supported by National Institutes of Health Training Grant T32 HL 07312.

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0046-8177/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.humpath.2006.09.016

of IE in the placenta. In endemic areas, peripheral blood smears are reported to be negative in roughly half of women with placental IE for unknown reasons [2]. The presence of placental IE defines active PM episodes. Past PM episodes are indicated by malarial pigment deposition in the absence of IE [3,4]. Active PM episodes can present histologically as acute (high parasite density with minimal inflammation and pigment) or as chronic (typically lower parasite density with extensive inflammation and pigment).

2. Materials and methods

Placental samples and clinical information were provided by Tanzanian women 18 to 45 years of age, delivering at the Muheza Designated District Hospital, Muheza, Tanga region. Sample donors were among those recruited to participate in a birth cohort study known locally as the Mother-Offspring Malaria Study. Women provided signed informed consent before joining the study. Study procedures involving human subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health, and ethical clearance was obtained from the institutional review board of Seattle Biomedical Research Institute and the National Medical Research Coordinating Committee in Tanzania.

Immediately before delivery, peripheral blood was collected. The placenta was collected at delivery, and a full-thickness biopsy from the middle third of the placental disk was frozen in liquid nitrogen and stored at −80°C. Active PM episodes were detected by microscopy of Giemsa-stained thick and thin smears of placental blood extracted from placental tissue by mechanical grinding. Placental parasitemia was quantified as the percentage of IE.

Cryosections (5 μm) of placental tissue were Giemsa-stained and assessed by examining greater than 100 60× fields per section. Malarial pigment is a brown heme-derived product that refracts under polarizing light and persists in acellular fibrinoid in the intervillous space. Past PM episodes were defined by the presence of pigment in a placenta negative for IE. Pigment deposition in fibrinoid was quantified by determining the percentage of fields containing pigment, presented as mean ± SD and analyzed using Student *t* test. Inflammation was qualitatively scored by the presence of inflammatory cells in the intervillous space.

DNA was extracted from 30-μL packed red blood cell pellets using Qiagen QIAmp DNA blood kit (Valencia, CA). To detect parasite DNA, nested polymerase chain reaction (PCR) for *P falciparum* 18s rRNA was performed. This method has a published sensitivity of 10 parasites per reaction [5]. The primer pair for the first amplification was TTAAAATTGTTGCAGTTAAAACG (forward) and CCTGTTGTTGCCTTAAACTTC (reverse); the primer pair for the second amplification was TTAAACTGGTTTGG-GAAAACCAAATATATT (forward) and ACACAAT-GAACTCAATCATGACTACCCGTC (reverse).

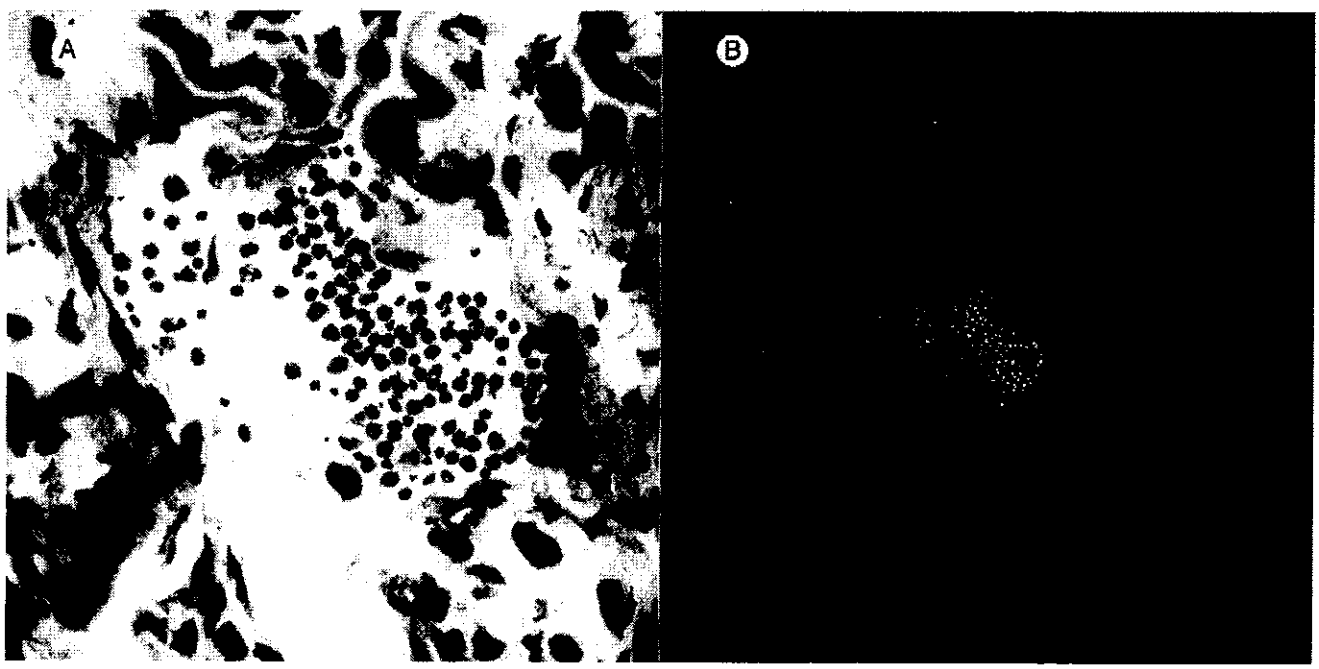

Fig. 1 A, Placental tissue cryosection showing a lone nidus of parasites (Giemsa, 600×). B, Polarized light showing malarial pigment and adjacent normal tissue (200×).

3. Results

A series of 132 first-time mothers delivering single viable offspring from September 2002 to June 2004 were examined. IE were observed by microscopy in the placental bloodsmears of 35 of 132 women. Placental parasitemia ranged from 0.03% to 74% IE. All cases had a uniform distribution of IE across intervillous spaces by histology. Peripheral IE were detected by microscopy in 24 of 35 women with placental IE.

Past PM episodes were identified by placental pigment deposition in 46 of 97 mothers negative for IE. Pigment deposition was greatest in women with active (28.4 ± 19.6% of fields; n = 33) compared with those with past PM episodes (18.0% ± 11.3% of fields; n = 39; $P < .007$). Likewise, intervillous inflammation was more common among women with active (26/35) compared with those with past PM episodes (8/46). Inflammatory cells were uniformly distributed across intervillous spaces in all cases.

The epidemiological pattern of PM that we observed was typical of malaria-endemic areas [6]. However, in 1 of 97 women negative for IE by placental bloodsmear, a single nidus of IE was observed in a single intervillous space by histology. Her case is reported below.

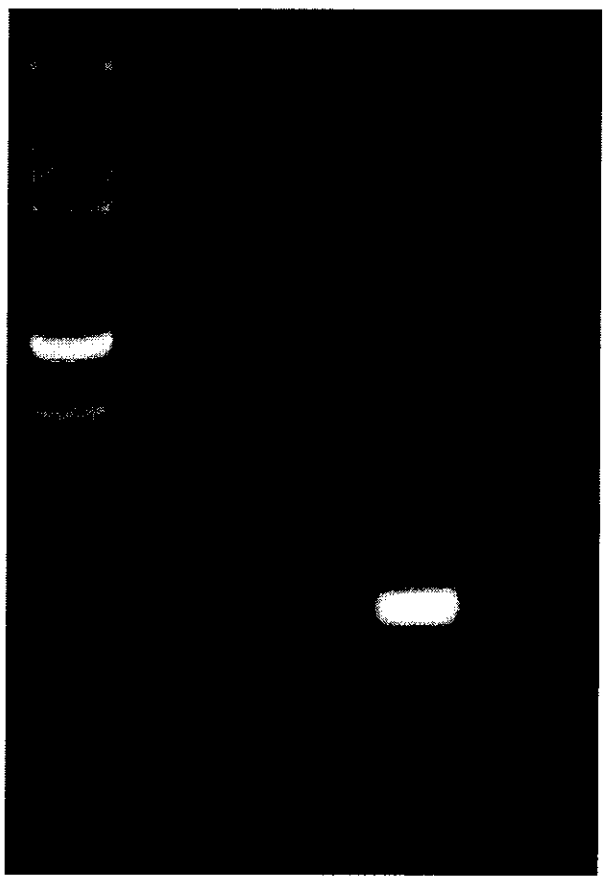

Fig. 2 Nested PCR for *P falciparum* 18s rRNA gene: 100-bp ladder (lane 1), the patient's peripheral (lane 2) and placental blood (lane 3), placental blood from an infected Tanzanian woman (lane 4), and peripheral blood from an uninfected subject (lane 5).

3.1. Case report

In January 2004, a 20-year-old primigravida presented for delivery. Her vital signs during admission for delivery were as follows: temperature, 36°C; pulse, 74 beats per minute; respiratory rate, 22 per minute; and blood pressure, 110/80 mm Hg. The patient reported receiving routine intermittent presumptive treatment during pregnancy, consisting of 2 doses of sulfadoxine-pyrimethamine, 1 each in the second and third trimester to prevent malaria. Spontaneous vaginal delivery of a 4.2-kg male infant was uneventful. The newborn developed neonatal conjunctivitis 2 weeks after delivery, which was successfully treated with antibiotics.

Giemsa-stained thin and thick smears of peripheral and placental blood samples were negative for IE by microscopy. However, histologic examination of 5-μm cryosection from a fresh frozen placental biopsy revealed a lone focus of 166 late trophozoite and early schizont (mature) parasites in an intervillous space (Fig. 1A). No additional parasites were observed in this or in 15 subsequent sections. Malaria pigment within fibrinoid was observed in 2.9% of fields, indicating a past PM episode during this pregnancy. Her level of pigment deposition was below the 10th percentile observed across all women with past infections. Inflammatory cells were absent in all fields examined. Polarized light demonstrated malarial pigment within the parasites; however, adjacent tissue did not have pigment and was histologically normal (Fig. 1B). Peripheral and placental blood samples from this woman did not yield a PCR-amplified 205-bp product using 2 μL of packed erythrocytes per reaction (Fig. 2).

4. Discussion

We observed a single persisting nidus of *P falciparum* IE in a first-time mother with histologic evidence of a past PM episode and a history of receiving presumptive malaria treatment during pregnancy. Low placental IE densities were also observed in other women, but in all those cases, IE were distributed evenly throughout the placenta without a localized nidus of sequestered IE.

The etiology of the lesion in this case is best explained as a clonal population derived from a single sequestered IE that multiplied through local reinvasion in a single intervillous space. However, we cannot exclude that a cluster of dormant IE persisted in this woman after clearance of other IE by drugs or immune responses. No similar case has been reported.

Our proposed etiology requires that ring-stage (immature) IE remain localized to a site of sequestration, which challenges the prevailing view that ring-stage IE necessarily circulate in the peripheral blood. Ring-stage IE have been observed to be sequestered in brain capillaries [7] and to adhere to mammalian cells in vitro [8]. In a study from Malawi, ring-stage IE were enriched in placental blood in

2 of 14 cases of PM [9]. In the MOMS Project, ring-stage IE have been the predominant IE form in 5 of 114 cases of active PM (data not shown). Our observations support the model proposed by Pouvelle et al [8] that a proportion of the IE causing PM adhere throughout development, which may account, in part, for the absence of peripheral IE observed by microscopy in a proportion of women with placental IE.

The single nidus of IE raises the intriguing possibility of a persistent or dormant blood-stage form of *P falciparum*. *P vivax* liver-stage hypnozoites can be dormant for up to 2 years, and *P ovale* also forms liver-stage hypnozoites. Blood-stage *P malariae* (which does not form liver-stage hypnozoites) may recrudesce decades after the initial infection [10], and the basis for this remarkable persistence of blood-stage parasitemia is unknown. Like *P malariae*, *P falciparum* does not form liver-stage hypnozoites, but a single infection can yield multiple recrudescences after intervening periods of subpatency. A latent form of merozoites (which normally invade erythrocytes) has been proposed to persist in the lymphatic network of rodents infected with *P yoelii*, *P chabaudi*, or *P vinckei* [11].

This woman had an extraordinarily low-density parasitemia—no IE were detected in peripheral or placental blood by either microscopy or by PCR amplification of parasite DNA. This case suggests *P falciparum* malaria may persist at densities as low as a single IE, which may initiate a recrudescence. In endemic areas, such low-density infections could act as a reservoir of malaria during periods when transmission by mosquitoes has ceased.

Acknowledgments

The authors thank the MOMS Project nurses who collected and processed the placental samples, and the Project microscopists who prepared and examined the placental bloodsmears.

References

[1] Keuter M, van Eijk A, Hoogstrate M, et al. Comparison of chloroquine, pyrimethamine and sulfadoxine, and chlorproguanil and dapsone as treatment for falciparum malaria in pregnant and non-pregnant women, Kakamega District, Kenya. BMJ 1990;301: 466-70.

[2] Mockenhaupt FP, Ulmen U, von Gaertner C, Bedu-Addo G, Bienzle U. Diagnosis of placental malaria. J Clin Microbiol 2002;40:306-8.

[3] Bulmer JN, Rasheed FN, Francis N, Morrison L, Greenwood BM. Placental malaria. I. Pathological classification. Histopathology 1993;22:211-8.

[4] Garnham PCC. The placenta in malaria with special reference to reticulo-endothelial immunity. Trans R Soc Trop Med Hyg 1938; 13-48.

[5] Snounou G, Viriyakosol S, Zhu XP, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993;61:315-20.

[6] Ismail MR, Ordi J, Menendez C, et al. Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. HUM PATHOL 2000;31:85-93.

[7] Silamut K, Phu NH, Whitty C, et al. A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am J Pathol 1999;155:395-410.

[8] Pouvelle B, Buffet PA, Lepolard C, Scherf A, Gysin J. Cytoadhesion of *Plasmodium falciparum* ring-stage–infected erythrocytes. Nat Med 2000;6:1264-8.

[9] Beeson JG, Amin N, Kanjala M, Rogerson SJ. Selective accumulation of mature asexual stages of *Plasmodium falciparum*–infected erythrocytes in the placenta. Infect Immun 2002;70:5412-5.

[10] Vinetz JM, Li J, McCutchan TF, Kaslow DC. Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. N Engl J Med 1998;338:367-71.

[11] Landau I, Chabaud AG, Mora-Silvera E, et al. Survival of rodent malaria merozoites in the lymphatic network: potential role in chronicity of the infection. Parasite 1999;6:311-22.

Genome-Wide Expression Analysis of Placental Malaria Reveals Features of Lymphoid Neogenesis during Chronic Infection[1]

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Chronic inflammation during placental malaria (PM) is most frequent in first time mothers and is associated with poor maternal and fetal outcomes. In the first genome-wide analysis of the local human response to sequestered malaria parasites, we identified genes associated with chronic PM and then localized the corresponding proteins and immune cell subsets in placental cryosections. B cell-related genes were among the most highly up-regulated transcripts in inflamed tissue. The B cell chemoattractant CXCL13 was up-regulated >1,000-fold, and B cell-activating factor was also detected. Both proteins were expressed by intervillous macrophages. Ig L and H chain transcription increased significantly, and heavy depositions of IgG3 and IgM were observed in intervillous spaces. The B cell phenotype was heterogenous, including naive (CD27-negative), mature (CD138-positive), and cycling (Ki-67-positive) cells. B cells expressed T-bet but not Bcl-6, suggesting T cell-independent activation without germinal center formation. Genes for the Fc binding proteins FcγRIa, FcγRIIIa, and C1q were highly up-regulated, and the proteins localized to intervillous macrophages. Birth weight was inversely correlated with transcript levels of CXCL13, IgG H chain, and IgM H chain. The iron regulatory peptide hepcidin was also expressed but was not associated with maternal anemia. The results suggest that B cells and macrophages contribute to chronic PM in a process resembling lymphoid neogenesis. We propose a model where the production of Ig during chronic malaria may enhance inflammation by attracting and activating macrophages that, in turn, recruit B cells to further produce Ig in the intervillous spaces. ***The Journal of Immunology,*** **2007, 179: 557–565.**

Placental malaria (PM)[3] due to *Plasmodium falciparum* is a major cause of death for mothers and their offspring, with the heaviest burden of disease occurring in first pregnancies. PM is caused by infected erythrocyte (IE) forms that bind to chondroitin sulfate A and sequester in the placenta (1).

Active PM episodes are defined by the presence of IE in the intervillous space. PM may present acutely with minimal inflammation or chronically with extensive inflammation and malarial pigment deposition (2). Chronic PM is most closely associated with maternal and fetal morbidity (3). Past PM episodes are defined by the persistence of malarial pigment (or hemozoin) in the absence of IE.

First time mothers commonly suffer chronic PM characterized by inflammatory infiltrates in the intervillous spaces and increased levels of type I cytokines (4) and β chemokines (5, 6). The intervillous infiltrate consists primarily of macrophages with a smaller number of T cells, B cells, and granulocytes (7). This infiltrate can sometimes become so extensive that it appears to occlude the maternal circulation, a condition called massive chronic intervillositis (8).

Women become resistant to PM over successive pregnancies as they acquire Abs that inhibit the binding of placental parasites to chondroitin sulfate A (9). These Abs are associated with a decreased risk of PM and increased birthweight and gestational age of the newborn (10). Abs that label laboratory parasites selected to bind chondroitin sulfate A also increase over successive pregnancies (11, 12), are elevated during infection (11), and have been associated with protection against anemia in first time mothers with chronic PM (13). First time mothers with active PM have elevated levels of total IgG and IgM (14), but these do not inhibit the binding of placental parasites (9) and this latter observation may explain the slow clearance of parasites from these women (15). In women without specific immunity, phagocytic cells may play a more prominent role in clearing parasites (16).

Abs can damage tissue. During type III hypersensitivity reactions as defined by Coombs and Gell (17), immune complexes deposit in tissue and activate phagocytic cells and complement. Immune complexes play a role in chronic inflammatory diseases such as rheumatoid arthritis (18) and Lyme arthritis (19), and the macrophage Fc receptor FcγRIIIa (CD16) is necessary in spontaneous murine autoimmune arthritis (20). Malaria has been associated with circulating immune complexes (21), and Ig and parasite

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Received for publication October 13, 2006. Accepted for publication April 3, 2007.

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[1] This work was supported by a grant from the Bill and Melinda Gates Foundation (to P.E.D.), National Institutes of Health Grant R01AI52059 (to P.E.D.), and funds from the American Medical Association Foundation and the Washington State Obstetrical Association (to A.M.). The gene expression arrays were funded by National Institutes of Health Grant HL072370. A.M. was supported by National Institutes of Health Training Grant T32 HL07312.

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[3] Abbreviations used in this paper: PM, placental malaria; BAFF, B cell-activating factor; C_T, threshold cycle; IE, infected erythrocyte.

Table I. *Primers used in this study*

Target	Primer Type	Primers	Product Size (bp)
IGGH (all isotypes)	Forward	CAAGTGCAAGGTCTCCAACA	138
	Reverse	AGGCTGACCTGGTTCTTGGT	
IGMH	Forward	ACCAGCACACTGACCATCAA	186
	Reverse	GGTGGACTTGGTGAGGAAGA	
IFNG	Forward	TGACCAGAGCATCCAAAAGA	147
	Reverse	TGTATTGCTTTGCGTTGGAC	
IL1B	Forward	CTGTCCTGCGTGTTGAAAGA	178
	Reverse	CTGCTTGAGAGCTGCTGATG	
IL18	Forward	TGCATCAACTTTGTGGCAAT	220
	Reverse	ATATGGTCCGGGGTGCATTA	
TNF	Forward	CACGCTCTTCTGCCTGCT	161
	Reverse	CAGCTTGAGGGTTTGCTACA	
BAFF	Forward	CGTTCAGGGTCCAGAAGAAA	115
	Reverse	AAAGCTGAGAAGCCATGGAA	
Hepcidin	Forward	GACCAGTGGCTCTGTTTTCC	193
	Reverse	CTACGTCTTGCAGCACATCC	
CXCL10	Forward	CCACGTGTTGAGATCATTGC	180
	Reverse	CCTCTGTGTGGTCCATCCTT	
CXCL9	Forward	GAAGCAGCCAAGTCGGTTAG	75
	Reverse	TGGAAGGAGGTTTCCACATC	
CXCL13	Forward	GGGAATGGTTGTCCAAGAAA	213
	Reverse	CAGAGCAGGGATAAGGGAAG	
CXCL16	Forward	GCCCTTTCCTATGTGCTGTG	121
	Reverse	AGCTTCCATTCTTGGCTCAG	
CCL5	Forward	CGCTGTCATCCTCATTGCTA	196
	Reverse	ACACACTTGGCGGTTCTTTC	
CCL4	Forward	CTTCCTCGCAACTTTGTGGT	88
	Reverse	GCTTGCTTCTTTTGGTTTGG	
CCL18	Forward	CCTGGCAGATTCCACAAAAG	126
	Reverse	CCCACTTCTTATTGGGGTCA	
KRT7	Forward	GGCTGAGATCGACAACATCA	103
	Reverse	CTTGGCACGAGCATCCTT	

Ag depositions have been observed in the basement membrane of endothelial cells during cerebral malaria (22) and in that of trophoblast during PM (23). Serum Ig is elevated during PM (14), yet the relationship between Ig and inflammation during PM is not known, nor has Fc receptor expression been examined in the abundant macrophages that accumulate in the placenta.

Lymphoid neogenesis is the process that results in the ectopic accumulation of lymphoid cells in chronically inflamed tissues. These cellular accumulations, known as tertiary lymphoid organs, have been observed in inflamed tissue resulting from autoimmunity, allograft rejection, and some microbial infections (24). Inflammatory cytokines, lymphoid chemokines, and various developmental stages of B cells are features of tertiary lymphoid organs during rheumatoid arthritis (25), Sjogren's syndrome (26), and Lyme borreliosis (27). The lymphoid chemokine CXCL13 is chemotactic for B cells expressing the Burkitt's lymphoma receptor CXCR5 and is essential for lymph node development in mice (28). CXCL13 expression has been associated with lymphoid neogenesis in autoimmune diseases (26, 27, 29) and during *Helicobacter pylori* and *Bartonella henselae* infection (30, 31). Although B cells contribute to the placental infiltrate during PM, no reports have characterized the B cells nor has CXCL13 been examined in malaria-infected individuals.

Genome-wide expression analysis of the host response during malaria infection has been examined in animal models of malaria infection (32, 33) and in human peripheral blood (34, 35). P.C.C. Garnham regarded the changes observed in the peripheral blood to be "merely a mild reflection of the real mechanism occurring in the internal organs" during *P. falciparum* infections (16). In this study we report the first genome-wide analysis of malaria-positive tissue in humans that reveals features of lymphoid neogenesis during chronic PM. The data suggest that macrophage CXCL13 expression, B cell recruitment, local Ab production, and Ab-mediated activation of phagocytes contribute to the pathogenesis of chronic placental malaria.

Table II. *Antibodies used in this study*

Host	Antigen	Dilution	Manufacturer
Mouse	FcγRIII (CD16)	1/500	Chemicon
Mouse	FcγRI (CD64)	1/500	Chemicon
Mouse	CD138	1/1,000	Chemicon
Mouse	CXCL13	1/50	R&D Systems
Mouse	BAFF	1/500	ID Labs
Mouse	IgG3-HRP	1/500	Zymed Laboratories
Goat	IgM-HRP	1/1,000	Chemicon
Rabbit	C1q	1/20,000	DakoCytomation
Rabbit	Hepcidin (hepc12A)	1/200	Alpha Diagnostics
Rabbit	CD79a	1/100	NeoMarkers
Mouse	CD27	1/50	Chemicon
Mouse	Ki-67	1/200	DakoCytomation
Mouse	T-bet	1/200	Santa Cruz Biotechnology
Mouse	Bcl-6	1/50	Chemicon
Mouse	Isotype (IgG1)	1/50	eBiosciences

Table III. *Clinical characteristics of women who donated samples examined for global gene expression*

	PM-Negative	PM-Positive	*p* Value
Age (years)	22.5 (3.0)	19.6 (1.8)	0.018
Infant weight (kg)	3.22 (0.31)	2.80 (0.26)	0.006
Female infants (*n*)	4/10	6/10	0.498
Placental parasite density (%)[a]	0	1.5 (0.6–63)	NA[b]
Pigment-positive (*n*)	5/10	10/10	NA[b]
Inflammation-positive (*n*)	1/10	7/10	0.01

[a] Percentage of IE.
[b] Not applicable.

Materials and Methods

Human subjects

Placental samples and clinical information were provided by Tanzanian women aged 18 to 45 years delivering at the Muheza Designated District

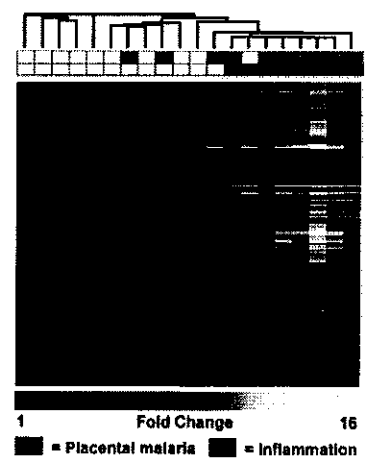

FIGURE 1. Hierarchical clustering of 728 probes associated with PM and hierarchical clustering of samples. Data are presented as fold change over the mean of PM-negative samples.

Table IV. *Expression data for selected genes up-regulated during inflammation (>2.5 fold change and $p < 0.01$)*

Gene Name	Gene Symbol	No. of Probes	*p* Value	Fold Change
Chemokine-related				
Chemokine (CXC motif) ligand 13	*CXCL13*	1	8.77E-08	129.3
Chemokine (CC motif) ligand 18	*CCL18*	2	2.53E-10	47.3
Chemokine (CXC motif) ligand 9	*CXCL9*	1	6.43E-09	32.0
Chemokine (CC motif) ligand 3	*CCL3*	1	6.03E-06	13.7
Chemokine (CC motif) ligand 4	*CCL4*	1	1.27E-04	13.5
Chemokine (CXC motif) ligand 10	*CXCL10*	1	1.56E-04	8.1
Chemokine (CX3C motif) receptor 1	*CX3CR1*	1	9.05E-04	5.5
Chemokine (CC motif) ligand 5	*CCL5*	3	2.25E-03	4.3
Chemokine (CXC motif) ligand 16	*CXCL16*	1	3.81E-07	4.1
Chemokine (CC motif) receptor 5	*CCR5*	1	1.72E-04	4.1
Chemokine (CC motif) receptor 1	*CCR1*	2	3.48E-03	2.8
Cytokine-related				
BAFF (TNF (ligand) superfamily, member 13b)	*TNFSF13B*	2	6.92E-09	8.7
IL-1R antagonist	*IL1RN*	1	5.44E-04	5.3
IL-10R α	*IL10RA*	1	3.79E-05	3.9
IL-1β	*IL1B*	1	2.49E-03	3.6
IL-18 (IFN-γ -inducing factor)	*IL18*	1	8.29E-05	3.4
TNF (ligand) superfamily, member 13	*TNFSF13*	1	7.91E-05	2.9
Caspase 1 (IL-1β, convertase)	*CASP1*	1	3.80E-03	2.6
Immunoglobulin				
Igλ locus	*IGL*	4	5.51E-06	33.0
Igκ locus	*IGK*	6	1.26E-05	25.0
Ig heavy locus	*IGH*	1	1.54E-04	11.8
Ig J polypeptide	*IGJ*	1	4.18E-04	8.1
Fc receptor				
Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	*FCGR3A*	1	8.98E-06	6.1
Fc fragment of IgE, high affinity I, receptor for; γ polypeptide	*FCER1G*	2	1.05E-04	4.3
Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	*FCGR3B*	1	1.36E-03	3.1
Fc fragment of IgG, high affinity Ia, receptor (CD64)	*FCGR1A*	2	1.46E-03	2.9
Complement				
Complement component 1, q subcomponent, β polypeptide	*C1QB*	1	3.82E-05	3.6
Complement component 3a receptor 1	*C3AR1*	1	6.51E-05	3.2
Complement component 3	*C3*	1	3.14E-04	3.0
Complement component 5 receptor 1 (C5a)	*C5R1*	1	4.00E-03	2.9
Complement component 1, q subcomponent, γ polypeptide	*C1QG*	1	4.06E-04	2.8
Complement component 1, q subcomponent, α polypeptide	*C1QA*	1	5.90E-04	2.7
B cell expressed				
Regulator of G protein signaling 1	*RGS1*	2	1.41E-06	11.9
Protein kinase C, β 1	*PRKCB1*	1	2.48E-06	8.6
CD48 Ag (B cell membrane protein)	*CD48*	1	1.95E-07	8.5
SAM domain, SH3 domain and nuclear localization signals, 1	*SAMSN1*	2	6.23E-05	6.9
CD72 Ag	*CD72*	1	4.84E-04	4.4
CD37 Ag	*CD37*	1	1.48E-04	3.9
Bruton agammaglobulinemia tyrosine kinase	*BTK*	1	1.58E-04	2.6
T cell expressed				
SLAM family member 7	*SLAMF7*	1	3.70E-05	11.4
Granzyme A (CTLA3)	*GZMA*	1	1.51E-04	7.1
TCR α locus	*TRA*	2	2.64E-04	6.8
TCR β locus	*TRB1*	2	1.05E-04	6.0
Pleckstrin homology, Sec7 and coiled-coil domains, binding protein	*PSCDBP*	1	6.00E-07	5.4
Fibrinogen-like 2	*FGL2*	1	2.58E-05	4.6
CD2 antigen	*CD2*	1	2.87E-03	4.2
TCR γ locus	*TRG*	2	3.05E-03	4.1
Granzyme B (CTLA1)	*GZMB*	1	5.34E-03	3.6

(*Table continues*)

Table IV. (*Continued*)

Gene Name	Gene Symbol	No. of Probes	*p* Value	Fold Change
Macrophage expressed				
Sialic acid binding Ig-like lectin 10	*SIGLEC10*	1	3.17E-10	21.6
ADAM-like, decysin 1	*ADAMDEC1*	1	9.50E-08	13.2
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 1	*EMR1*	1	6.15E-07	12.8
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 2	*EMR2*	1	2.35E-06	7.4
SLAM family member 8 (BLAME)	*SLAMF8*	2	5.18E-06	5.7
CD86 antigen (CD28 antigen, B7-2 antigen)	*CD86*	1	5.35E-05	5.3
C-type lectin domain family 7, member A	*CLEC7A*	1	1.38E-04	4.3
macrophage receptor with collagenous structure	*MARCO*	1	1.29E-04	3.9
CD163 Ag	*CD163*	2	3.53E-04	3.8
Myeloid cell nuclear differentiation antigen	*MNDA*	1	3.84E-04	3.2
Macrophage expressed gene 1	*MPEG1*	2	4.99E-03	3.1
Ficolin 1	*FCN1*	1	3.75E-03	3.1
Chitinase 3-like 1	*CHI3L1*	1	1.93E-03	3.0
Antigen presentation				
MHC, class II, DQ α 1	*HLA-DQA1*	3	1.42E-07	37.4
MHC class II, DP β 1	*HLA-DPB1*	1	1.45E-08	14.0
MHC class II, DP α 1	*HLA-DPA1*	2	3.62E-07	12.3
MMHC, class II, DM α	*HLA-DMA*	1	2.55E-07	11.8
MHC class II, DQ β 1	*HLA-DQB1*	6	1.08E-04	10.9
MHC, class II, DR α	*HLA-DRA*	2	2.65E-08	10.2
MHC, class II, DR β 1	*HLA-DRB1*	4	5.73E-08	10.1
Proteasome subunit, β type, 9	*PSMB9*	1	1.31E-05	6.0
Cathepsin S	*CTSS*	3	3.74E-06	7.9
MHC, class II, DR β 3	*HLA-DRB5*	1	3.66E-07	6.6
CD74 antigen	*CD74*	2	1.09E-06	5.8
MHC, class II, DM β	*HLA-DMB*	1	6.57E-06	3.3
Pattern recognition				
TLR-8	*TLR8*	2	1.10E-07	12.6
Formyl peptide receptor-like 2	*FPRL2*	2	5.24E-08	12.2
TLR-1	*TLR1*	1	5.61E-06	5.8
TLR-2	*TLR2*	1	1.58E-05	5.4
Formyl peptide receptor 1	*FPR1*	1	5.46E-04	3.3
TLR-4	*TLR4*	2	1.12E-03	2.9
Redox related				
Superoxide dismutase 2, mitochondrial	SOD2	2	3.95E-06	7.9
Cytochrome b-245, β polypeptide	*CYBB*	1	2.02E-04	4.4
Cytochrome b-245, α polypeptide	*CYBA*	1	2.66E-05	2.8
Heme oxygenase (decycling) 1	*HMOX1*	1	2.16E-04	2.6
Intercellular adhesion				
Epidermal growth factor-like module containing, mucin-like, hormone receptor-like 2	*EMR2*	1	2.35E-06	7.4
Integrin, β_2 (Ag CD18)	*ITGB2*	2	1.27E-06	6.0
Integrin, α_L (Ag CD11A)	*ITGAL*	1	1.78E-04	4.1
Other				
Serpin peptidase inhibitor, clade A1	*SERPINA1*	2	2.50E-08	11.3
Adenosine deaminase	*ADA*	2	1.53E-06	7.2
Lysozyme (renal amyloidosis)	*LYZ*	2	3.14E-04	7.1
Prostaglandin E receptor 4 (subtype EP4)	*PTGER4*	1	2.46E-06	6.2
Hepcidin antimicrobial peptide	*HAMP*	1	2.30E-05	4.0
Leukotriene A4 hydrolase	*LTA4H*	1	2.35E-05	3.3
Ferritin, heavy polypeptide 1	*FTH1*	1	8.12E-07	2.8

Hospital, Muheza, Tanga region, in an area of intense malaria transmission. These women were participating in a birth cohort study known locally as the Mother-Offspring Malaria Study. Women signed an informed consent form before joining the study, and those with chronic debilitating disease were excluded. Clinical information was collected by project nurses and assistant medical officers on standardized forms. Study procedures involving human subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health, and ethical clearance was obtained from the institutional review boards of Seattle Biomedical Research Institute (Seattle, WA) and the National Institute for Medical Research in Tanzania.

Sample processing

The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was made. Tissue was fresh frozen in liquid nitrogen or collected in RNAlater (Ambion) and stored at −80°C. Placental blood was extracted from placental tissue by mechanical grinding. Placental malaria was diagnosed by microscopy of Giemsa-stained thick and thin smears of placental blood.

Table V. *Validation of array data by quantitative RT-PCR*

Gene	PM-negative (n = 22) compared with PM-Positive (19) Fold change	p Value	PM-Positive with (++) Intervillositis (5) Fold Change	p Value
CXCL13	46	<0.001	1242	<0.001
CCL18	11	<0.001	67	<0.001
IGGH	3	0.007	51	<0.001
HEPC	5.3	<0.001	38	<0.001
IFNG	4.2	0.001	37	<0.001
IGMH	2.2	0.008	34	<0.001
TNF	5.1	<0.001	19	<0.001
CCL4	4.5	<0.001	17	<0.001
CXCL9	2.9	0.006	14	<0.001
IL1B	4.3	<0.001	13	<0.001
CCL5	1.5	0.034	9.4	<0.001
IL18	2.1	<0.001	8.4	<0.001
CXCL10	1.8	0.062	8.2	0.003
BAFF	1.8	0.006	6.1	<0.001
CXCL16	1.9	<0.001	3.5	<0.001

Placental histopathology

For histologic analysis, 5-μm cryosections of placental tissue were fixed in methanol and Giemsa stained. Sections were assessed by examining >90 fields per section at ×60 magnification. Hemozoin deposition in fibrinoid was quantified by determining the proportion of fields with hemozoin present. Immune infiltrates within the intervillous spaces were qualitatively scored as negative (−) for none or very few inflammatory cells present, positive (+) for inflammatory cells present, and double positive (++) for having an extensive accumulation of inflammatory cells, i.e., massive chronic intervillositis.

Microarray analysis

For microarray analyses, placental villi were dissected at <0.5 mm^3, excluding large vessels, stem villi, infarcts, fetal membranes, or decidua from RNAlater-preserved placental tissues. Total RNA was extracted using RNeasy mini kits (Qiagen). RNA quality was assessed by an Agilent 2100 bioanalyzer, resulting in 28- to 18-s ratios of 1.1 to 1.5. Microarray assays were performed at the Center for Expression Arrays at the University of Washington (Seattle, WA). Biotinylated target cRNA was prepared and hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChip with minor modifications from the procedures recommended by Affymetrix. First-strand cDNA was produced by 5 μg of total RNA using a T7-linked oligo(dT) primer. In vitro transcription reaction was performed using biotinylated UTP and CTP. Fifteen micrograms of cRNA was fragmented, a hybridization mixture was assembled with the addition of spike-in controls, and chips were hybridized for 16 h. The chips were then washed and stained with streptavidin-PE using the Affymetrix GeneChip system and scanned using the GeneChip scanner. Transcription profiles were defined by GeneChip operating system (GCOS) absolute expression analysis. Data were normalized by the GeneChip robust multiarray analysis (GC-RMA) algorithm and then analyzed by t test and hierarchical clustering with Acuity 4.0 (Axon).

Quantitative RT-PCR

For quantitative PCR, total RNA was extracted from frozen cryosections using RNeasy mini kits (Qiagen). The RNA quality of representative

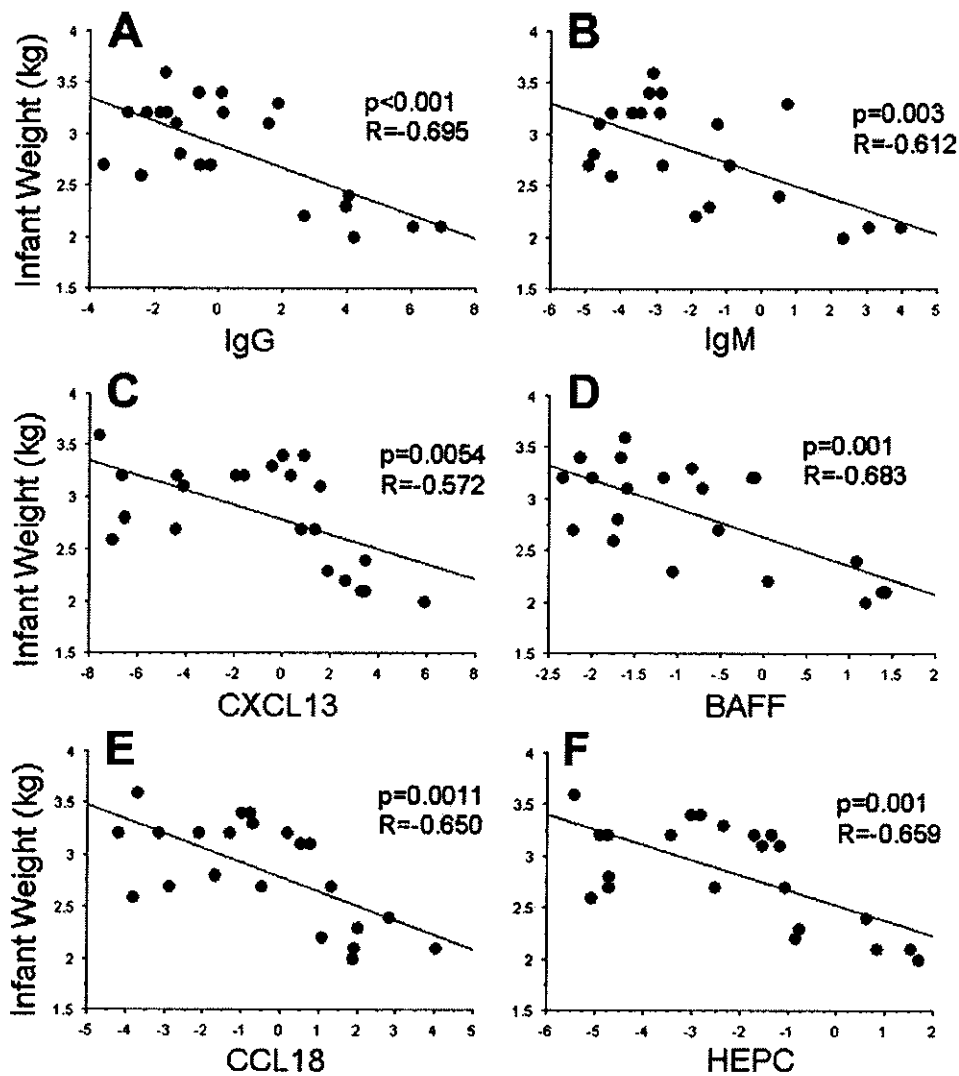

FIGURE 2. Correlation of gene expression by quantitative RT-PCR with birth weights for infected women. Gene expression for IgG (*A*), IgM (*B*), CXCL13 (*C*), BAFF (*D*), CCL18 (*E*), and hepcidin (HEPC) (*F*) is presented as $\log_2$-fold expression over KRT7. Simple regression analysis was used to calculate R and p values.

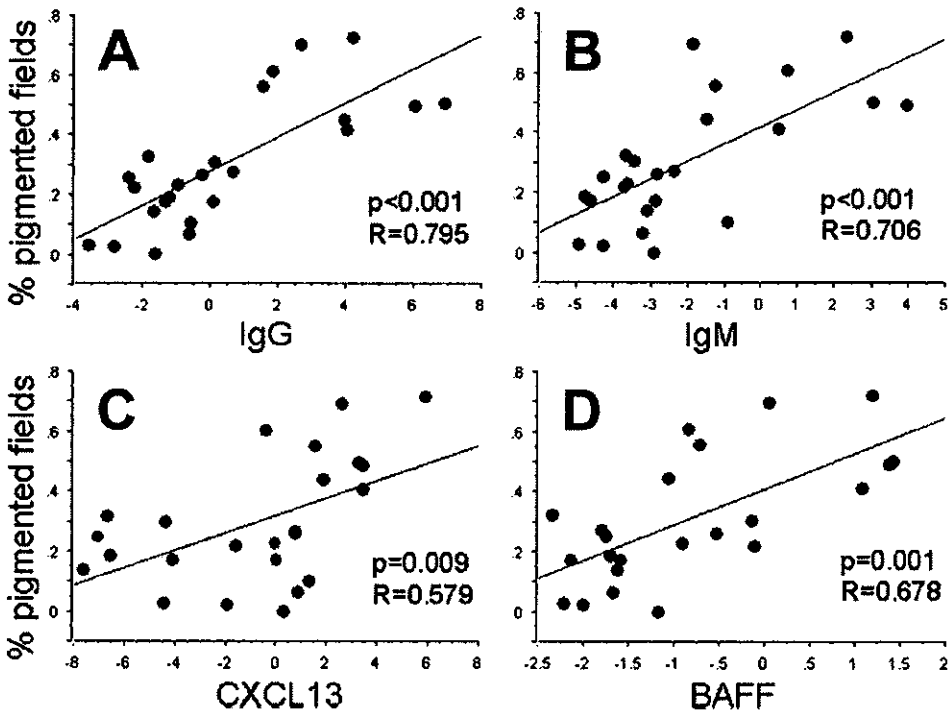

FIGURE 3. Correlation of gene expression by quantitative RT-PCR to placental malarial pigment deposition (proportion positive fields) by microscopy. Only infected women were analyzed. Gene expression for IgG (*A*), IgM (*B*), CXCL13 (*C*), and BAFF (*D*) is presented as 2-fold expression over KRT7. Simple regression analysis was used to calculate *R* and *p* values.

samples was assessed by an Agilent 2100 bioanalyzer, resulting in 28- to 18-s ratios of 1.1 to 1.5. cDNA was synthesized using a SuperScript III enzyme (Invitrogen Life Technologies) and anchored oligo$(dT)_{20}$ primers. Real-time PCR was performed in duplicate using SYBR Green Master Mix and an ABI Prism 7000 or 7500 system (Applied Biosystems). The annealing temperature was 60°C. Intron-spanning primers (except those for CXCL9, which comprise a single exon) were designed using Primer3 (Massachusetts Institute of Technology, Cambridge, MA). Primers for TNF were a gift from A. Collie (University of Washington, Seattle, WA). Primers are listed in Table I. All primers yielded single products, and amplification was linear on serial dilutions of cDNA samples. Threshold cycles (C_T) were calculated and normalized to the C_T of *KRT7* (a gene expressed by the trophoblast, not inflammatory cells) and *t* tests were performed on normalized C_T values. Data is presented as the fold difference from the control gene, calculated by $2^{(\text{control } C_T - \text{gene } C_T)}$.

Immunohistochemistry

For immunohistochemistry, 5-μm cryosections were fixed for 10 min in 4% paraformaldehyde (for CXCL13, IgG3, IgM, C1q, and hepcidin) or acetone (for CD16, CD64, CD138, and B cell activating factor (BAFF)). Abs and dilutions are listed in Table II. Indirect staining was performed using an anti-mouse or anti-rabbit diaminobenzidine (DAB) EnVision+ kit (DakoCytomation) according to manufacturer's directions. Direct staining was performed for IgG isotypes and IgM on sections blocked with species-concordant serum. For immunofluorescence studies, tissue was fixed for 10 min in 4% paraformaldehyde and then Alexa Fluor 488 chicken anti-mouse (Molecular Probes) or tetramethylrhodamine isothiocyanate goat anti-rabbit (Sigma-Aldrich) were used as secondary Abs. The sections were stained with 4′,6′-diamidino-2-phenylindole (Sigma-Aldrich) to define nuclei, mounted in 80% glycerol, and visualized using a fluorescent microscope.

Results

For global gene expression analysis, placental samples from 20 first time mothers were selected based on PM status and RNA quality. Ten had active PM episodes, and of the ten PM-negative women five had evidence of a past PM episode. Clinical characteristics of the women are summarized in Table III.

A normalized dataset was generated. Hierarchical clustering was performed to identify coregulated genes. We detected a group of 752 probes (correlation coefficient, 0.870) representing 528 coexpressed genes (Fig. 1) that were related to PM status. These included genes previously identified to be involved in PM such as *IFNG*, *TNF*, *CCL3*, *CCL4*, and *CCL5*. In analysis limited to these 752 probes, hierarchical clustering separated the samples from women with placental inflammation from those without placental inflammation (Fig. 1). Samples from three women with active PM but minimal inflammation clustered with PM-negative samples. In addition, one sample from a woman with a past PM episode possessed inflammatory cells and clustered with the active PM inflamed samples. The placental blood smear from this subject was re-examined and confirmed to be negative for IE. Samples from the other four women with past PM episodes clustered with the other PM-negative samples. No coexpressed genes were identified that correlated with percentage of IE or with the levels of malaria pigment deposition. These data suggest that the transcriptional changes observed during PM are more closely related to placental inflammation than to parasitemia or pigment deposition.

Statistical testing for differences in gene expression between seven PM-positive women with inflammation vs nine PM-negative women without inflammation revealed 314 probes representing 234 genes that were at least 2.5-fold elevated with $p < 0.01$ (see supplemental table).[4] For each of these probes, mean intensity was above the 25th percentile of all probe intensities. Probe data for IFN-γ and TNF did not meet these criteria. The most up-regulated gene was *CXCL13* at 130-fold, followed by *CCL18* at 47-fold, *HLADQA1* at 37-fold, *Igλ* at 33-fold, *CXCL9* at 32-fold, and *Igκ* at 25-fold. Genes associated with the immune response, particularly with B cell, T cell and macrophage function were identified. Selected genes are listed in Table IV.

We validated the expression of a subset of genes by quantitative RT-PCR over a larger group of samples (Table V). Primers for the IgG H chain were designed to amplify all IgG classes. The level of gene expression correlated with the level of inflammation for all genes analyzed, including *TNF* and *IFNG*. Remarkably, CXCL13

[4] The online version of this article contains supplemental material.

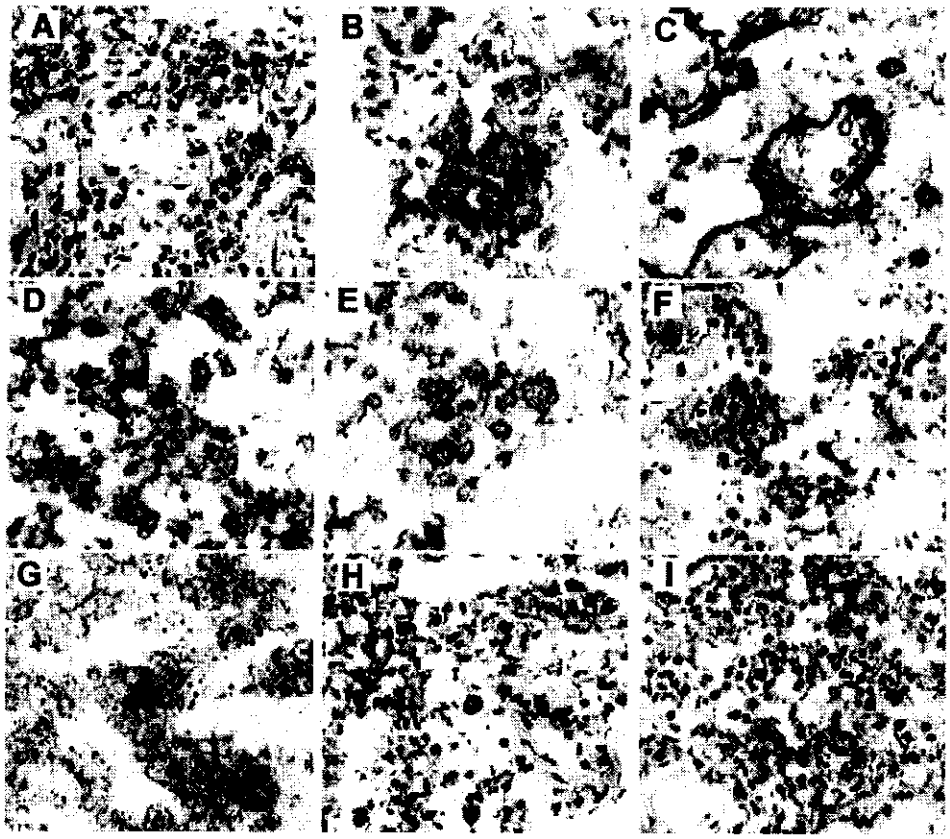

FIGURE 4. Immunohistochemistry of inflamed PM-positive tissues for IgM (*A*), IgG3 (*B*), CD138, a plasma cell marker (*C*), FcγRIII (CD16) (*D*), FcγRI (CD64) (*E*), C1q (*F*), BAFF (*G*), CXCL13 (*H*), and hepcidin (*I*). All fields are ×200 (original magnification).

was >1,000-fold up-regulated in the placentas of women with massive chronic intervillositis. Of the genes examined by quantitative RT-PCR, *CXCL13*, *IGGH*, and *IGMH* were disproportionately increased (27-, 17-, and 15-fold, respectively) in massive chronic intervillositis compared with other PM-positive samples.

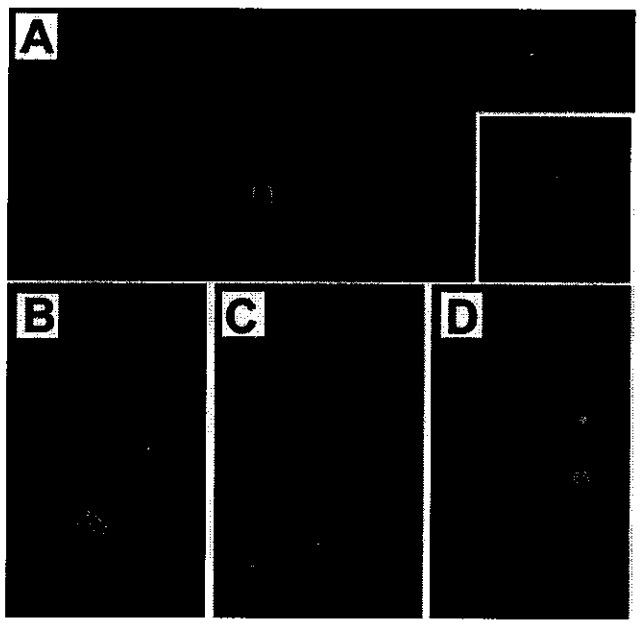

FIGURE 5. B cell phenotype analysis by double indirect immunofluorescence using CD79a to identify B cells (red), a second marker (green), and 4′,6′-diamidino-2-phenylindole to define nuclear DNA (blue). *A* and *inset*, CD27. *B*, Ki-67. *C*, T-bet. *D*, Bcl-6. All fields are ×400 (original magnification).

Several genes, including *IgG*, *IgM*, *BAFF*, *CXCL13*, *CCL18*, and hepcidin were negatively correlated with birthweight in PM-positive women (Fig. 2). Maternal hemoglobin did not correlate significantly with the placental hepcidin transcript level ($R = -0.143$, $p = 0.392$, $n = 38$). Several genes, notably *IgG*, *IgM*, *CXCL13*, and *BAFF*, were correlated with the degree of malarial pigment deposition (Fig. 3), a pathologic feature that reflects the chronicity of infection in PM-positive women.

By immunohistochemistry, IgG3 and IgM stained strongly in the intervillous space of PM-positive placentas and were associated with macrophages (Fig. 4). IgG1 and IgG4 levels were not elevated and IgG2 was moderately elevated (data not shown). Infiltrating macrophages stained positively for the Fc receptors FcγRIIIa (CD16), FcγRIa (CD64), and C1q. Macrophages also stained positively for CXCL13 and BAFF. Because of the high level of Ig transcription we tested for plasma cells and identified CD138-positive cells present in the intervillous space during chronic placental malaria, but not in uninfected women. The villous trophoblast also stained positive for CD138, a marker also expressed by epithelial cells including trophoblasts (36).

We further characterized the B cell population in chronic PM using double indirect immunofluorescence (Fig. 5). B cells were identified using CD79a. The majority of B cells were CD27 negative suggesting a naive phenotype, although CD27-positive cells were present (Fig. 5*A*, *inset*). Furthermore, a population of B cells expressed the mitotic marker Ki-67. The transcription factor T-bet was also expressed by a subset of B cells. B cells did not express Bcl-6, although it was observed in other cell types during chronic PM.

Discussion

The inflammatory response to sequestered *P. falciparum* parasites is thought to play a key role in the development of severe malaria syndromes. This is the first study to examine the transcriptome of

the local response to sequestered parasites in humans. The results highlight a hitherto unappreciated role for B cells during episodes of chronic inflammatory PM that echo features of lymphoid neogenesis, including macrophage CXCL13 expression, the accumulation of naive B cells, cycling B cells, abundant plasma cells, and IgM and IgG synthesis.

Malaria infection is associated with B cell pathology, including hypergammaglobulinemia (37), autoantibody production (38), and circulating immune complexes (21, 39). Malaria also has a strong geographic overlap with Burkitt's lymphoma, a B cell neoplasm (40). Hyperreactive malarial splenomegaly is marked by elevated levels of IgM and antimalarial Abs (41), and total serum Ig levels (14) and immune complex deposition (23) increase during PM. The mechanism of B cell dysfunction during PM may involve the accumulation of CD27-negative naive B cells in the placenta and their subsequent activation to produce nonspecific Abs. Activation may involve a T cell-independent Ig class switch, as evidenced by T-bet expression (42). In our study, B cells were not aggregated in follicular structures and did not express the germinal center marker Bcl-6. Similarly, T-bet expression but not Bcl-6 expression has been observed in B cell infiltrates of *B. henselae* granulomas (31).

Our data suggest that Ig and macrophages interact to contribute to the pathology in first time mothers with chronic PM. We propose a model in which *P. falciparum* Ag-Ab complexes in the intervillous space activate monocytes through Fcγ receptors (CD16 and CD64) and complement C1q. Macrophages stained positively for IgG3 and IgM, suggesting phagocytic uptake. Macrophage expression of CXCL13 and BAFF may contribute to B cell accumulation and Ig synthesis. We speculate that further Ig synthesis activates additional monocytes, thus generating a proinflammatory feedback loop. Such a proinflammatory feedback loop, involving B cells and macrophages, may be a general phenomenon during malaria infection because hypergammaglobulinemia and circulating immune complexes are also features of malaria in nonpregnant individuals. Although our data suggest a pathologic role for the B cell infiltrate during severe malaria, they do not exclude the possibility that these B cells may eventually lead to parasite clearance.

We observed the up-regulation of several genes, in addition to *CXCL13*, that are associated with chronic inflammation. Elevated levels of the chemokines CCL18 (43), CXCL16 (44), and CXCL9 (45) have also been observed in tertiary lymphoid organs and may recruit lymphocytes to the site of inflammation. Like CXCL13, CCL18 is expressed in germinal centers and attracts naive lymphocytes (43). The chemokines CXCL16 and CXCL9 attract plasma cells (46, 47). BAFF promotes B cell survival and its overexpression in mice leads to hypergammaglobulinemia and autoantibody production (48). IL1b and IL18 are primary drivers of inflammation in mice, are elevated during chronic autoimmune diseases of humans, and are therapeutic targets in rheumatoid arthritis (49, 50).

The type I cytokines TNF and IFN-γ are involved in the immune response to PM (4). TNF regulates CXCL13 expression in some models and may be upstream of CXCL13 during PM. TNF is necessary for experimental follicle formation (51) and stimulates dendritic cell CXCL13 production (52). We also observed evidence of type I differentiation in B cells. IgG3 was the predominant IgG isotype observed, and a subset of B cells expressed T-bet, which is induced by IFN-γ and is necessary for type I differentiation (53).

The pathways identified in this study should be examined in other severe malaria syndromes, as they may be general phenomena during malaria infection. In addition, multiple soluble molecules that we identified by microarray analysis have potential use as biomarkers for diagnosing or assessing the severity of *P. falciparum* infections. Hepcidin is a cytokine-induced peptide that is a key mediator of the anemia of inflammation (54). We detected hepcidin expression in intervillous macrophages during chronic malaria infection, although in the present study hepcidin was not associated with hemoglobin concentration.

In summary, our results suggest that macrophage CXCL13 expression, B cell recruitment, Ab synthesis, and Ab-mediated activation of phagocytes contribute to the pathogenesis of chronic placental malaria, echoing features of lymphoid neogenesis. These findings may explain how malaria causes B cell dysfunction, as well as the high levels of Ig that develop during chronic infection. Future studies should assess whether the activation of immature B cells at the site of sequestered parasites may also interfere with the acquisition of protective immunity to malaria.

Acknowledgments

Mother-Offspring Malaria Study (MOMS) Project nurses processed the samples used in these studies, and MOMS Project technicians interpreted the blood smears. Gene expression arrays were performed at the University of Washington Center for Expression Arrays (Seattle, WA) by K. Serikawa and R. Hall. K. Kerr and C. Wei (University of Washington, Seattle, WA) contributed to statistical analysis. We thank L. Guilbert (University of Alberta, Edmonton, Canada), S. Schwartz, D. Sabath, T. Easterling, D. Carr, and A. Craxton (University of Washington, Seattle, WA) and Jason Wendler (Seattle Biomedical Research Institute, Seattle, WA) for discussion.

Disclosures

The authors have no financial conflict of interest.

References

1. Fried, M., and P. E. Duffy. 1996. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* 272: 1502–1504.
2. Bulmer, J. N., F. N. Rasheed, N. Francis, L. Morrison, and B. M. Greenwood. 1993. Placental malaria, I: pathological classification. *Histopathology* 22: 211–218.
3. Duffy, P. E. 2001. Immunity to malaria during pregnancy: different host, different parasite. In *Malaria in Pregnancy: Deadly Parasite, Susceptible Host.* P. E. Duffy, and M. Fried, eds. Taylor and Francis, London, pp. 71–126.
4. Fried, M., R. O. Muga, A. O. Misore, and P. E. Duffy. 1998. Malaria elicits type 1 cytokines in the human placenta: IFN-γ and TNF-α associated with pregnancy outcomes. *J. Immunol.* 160: 2523–2530.
5. Abrams, E. T., H. Brown, S. W. Chensue, G. D. Turner, E. Tadesse, V. M. Lema, M. E. Molyneux, R. Rochford, S. R. Meshnick, and S. J. Rogerson. 2003. Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated β chemokine expression. *J. Immunol.* 170: 2759–2764.
6. Suguitan, A. L., Jr., R. G. Leke, G. Fouda, A. Zhou, L. Thuita, S. Metenou, J. Fogako, R. Megnekou, and D. W. Taylor. 2003. Changes in the levels of chemokines and cytokines in the placentas of women with *Plasmodium falciparum* malaria. *J. Infect. Dis.* 188: 1074–1082.
7. Ordi, J., C. Menendez, M. R. Ismail, P. J. Ventura, A. Palacin, E. Kahigwa, B. Ferrer, A. Cardesa, and P. L. Alonso. 2001. Placental malaria is associated with cell-mediated inflammatory responses with selective absence of natural killer cells. *J. Infect. Dis.* 183: 1100–1107.
8. Ordi, J., M. R. Ismail, P. J. Ventura, E. Kahigwa, R. Hirt, A. Cardesa, P. L. Alonso, and C. Menendez. 1998. Massive chronic intervillositis of the placenta associated with malaria infection. *Am. J. Surg. Pathol.* 22: 1006–1011.
9. Fried, M., F. Nosten, A. Brockman, B. J. Brabin, and P. E. Duffy. 1998. Maternal antibodies block malaria. *Nature* 395: 851–852.
10. Duffy, P. E., and M. Fried. 2003. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. *Infect. Immun.* 71: 6620–6623.
11. Beeson, J. G., E. J. Mann, S. R. Elliott, V. M. Lema, E. Tadesse, M. E. Molyneux, G. V. Brown, and S. J. Rogerson. 2004. Antibodies to variant surface antigens of *Plasmodium falciparum*-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J. Infect. Dis.* 189: 540–551.
12. O'Neil-Dunne, I., R. N. Achur, S. T. Agbor-Enoh, M. Valiyaveettil, R. S. Naik, C. F. Ockenhouse, A. Zhou, R. Megnekou, R. Leke, D. W. Taylor, and D. C. Gowda. 2001. Gravidity-dependent production of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy. *Infect. Immun.* 69: 7487–7492.
13. Staalsoe, T., C. E. Shulman, J. N. Bulmer, K. Kawuondo, K. Marsh, and L. Hviid. 2004. Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated *Plasmodium falciparum* malaria. *Lancet* 363: 283–289.

14. Logie, D. E., I. A. McGregor, D. S. Rowe, and W. Z. Billewicz. 1973. Plasma immunoglobulin concentrations in mothers and newborn children with special reference to placental malaria: studies in the Gambia, Nigeria, and Switzerland. *Bull. W. H. O.* 49: 547–554.
15. Brabin, B. J., and S. J. Rogerson. 2001. The epidemiology and outcomes of maternal malaria. In *Malaria in Pregnancy: Deadly Parasite, Susceptible Host.* P. E. Duffy, and M. Fried, eds. Taylor and Francis, London, pp. 27–52.
16. Garnham, P. C. C. 1938. The placenta in malaria with special reference to reticulo-endothelial immunity. *Trans R. Soc. Trop. Med. Hyg.* 13–48.
17. Gell, P. G. H., and R. R. A. Coombs. 1963. *Clinical Aspects of Immunology.* Blackwell, Oxford, p. 317–337.
18. Firestein, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature* 423: 356–361.
19. Hardin, J. A., L. C. Walker, A. C. Steere, T. C. Trumble, K. S. Tung, R. C. Williams, Jr., S. Ruddy, and S. E. Malawista. 1979. Circulating immune complexes in Lyme arthritis: detection by the 125I-C1q binding, C1q solid phase, and Raji cell assays. *J. Clin. Invest.* 63: 468–477.
20. Ji, H., K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, et al. 2002. Arthritis critically dependent on innate immune system players. *Immunity* 16: 157–168.
21. Adam, C., M. Geniteau, M. Gougerot-Pocidalo, P. Verroust, J. Lebras, C. Gibert, and L. Morel-Maroger. 1981. Cryoglobulins, circulating immune complexes, and complement activation in cerebral malaria. *Infect. Immun.* 31: 530–535.
22. Oo, M. M., M. Aikawa, T. Than, T. M. Aye, P. T. Myint, I. Igarashi, and W. C. Schoene. 1987. Human cerebral malaria: a pathological study. *J. Neuropathol. Exp. Neurol.* 46: 223–231.
23. Maeno, Y., R. W. Steketee, T. Nagatake, T. Tegoshi, R. S. Desowitz, J. J. Wirima, and M. Aikawa. 1993. Immunoglobulin complex deposits in *Plasmodium falciparum*-infected placentas from Malawi and Papua New Guinea. *Am. J. Trop. Med. Hyg.* 49: 574–580.
24. Drayton, D. L., S. Liao, R. H. Mounzer, and N. H. Ruddle. 2006. Lymphoid organ development: from ontogeny to neogenesis. *Nat. Immunol.* 7: 344–353.
25. Weyand, C. M., and J. J. Goronzy. 2003. Ectopic germinal center formation in rheumatoid synovitis. *Ann. NY Acad. Sci.* 987: 140–149.
26. Salomonsson, S., P. Larsson, P. Tengner, E. Mellquist, P. Hjelmstrom, and M. Wahren-Herlenius. 2002. Expression of the B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjogren's syndrome. *Scand. J. Immunol.* 55: 336–342.
27. Narayan, K., D. Dail, L. Li, D. Cadavid, S. Amrute, P. Fitzgerald-Bocarsly, and A. R. Pachner. 2005. The nervous system as ectopic germinal center: CXCL13 and IgG in lyme neuroborreliosis. *Ann. Neurol.* 57: 813–823.
28. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406: 309–314.
29. Shi, K., K. Hayashida, M. Kaneko, J. Hashimoto, T. Tomita, P. E. Lipsky, H. Yoshikawa, and T. Ochi. 2001. Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. *J. Immunol.* 166: 650–655.
30. Mazzucchelli, L., A. Blaser, A. Kappeler, P. Scharli, J. A. Laissue, M. Baggiolini, and M. Uguccioni. 1999. BCA-1 is highly expressed in *Helicobacter pylori*-induced mucosa-associated lymphoid tissue and gastric lymphoma. *J. Clin. Invest.* 104: R49–R54.
31. Vermi, W., F. Facchetti, E. Riboldi, H. Heine, S. Scutera, S. Stornello, D. Ravarino, P. Cappello, M. Giovarelli, R. Badolato, et al. 2006. Role of dendritic cell-derived CXCL13 in the pathogenesis of *Bartonella henselae* B-rich granuloma. *Blood* 107: 454–462.
32. Schaecher, K., S. Kumar, A. Yadava, M. Vahey, and C. F. Ockenhouse. 2005. Genome-wide expression profiling in malaria infection reveals transcriptional changes associated with lethal and nonlethal outcomes. *Infect. Immun.* 73: 6091–6100.
33. Sexton, A. C., R. T. Good, D. S. Hansen, M. C. D'Ombrain, L. Buckingham, K. Simpson, and L. Schofield. 2004. Transcriptional profiling reveals suppressed erythropoiesis, up-regulated glycolysis, and interferon-associated responses in murine malaria. *J. Infect. Dis.* 189: 1245–1256.
34. Griffiths, M. J., M. J. Shafi, S. J. Popper, C. A. Hemingway, M. M. Kortok, A. Wathen, K. A. Rockett, R. Mott, M. Levin, C. R. Newton, et al. 2005. Genomewide analysis of the host response to malaria in Kenyan children. *J. Infect. Dis.* 191: 1599–1611.
35. Ockenhouse, C. F., W. C. Hu, K. E. Kester, J. F. Cummings, A. Stewart, D. G. Heppner, A. E. Jedlicka, A. L. Scott, N. D. Wolfe, M. Vahey, and D. S. Burke. 2006. Common and divergent immune response signaling pathways discovered in peripheral blood mononuclear cell gene expression patterns in presymptomatic and clinically apparent malaria. *Infect. Immun.* 74: 5561–5573.
36. Jokimaa, V., P. Inki, H. Kujari, O. Hirvonen, E. Ekholm, and L. Anttila. 1998. Expression of syndecan-1 in human placenta and decidua. *Placenta* 19: 157–163.
37. McGregor, I. A., H. M. Gilles, J. H. Walters, A. H. Davies, and F. A. Pearson. 1956. Effects of heavy and repeated malarial infections on Gambian infants and children; effects of erythrocytic parasitization. *Br. Med. J.* 32: 686–692.
38. Greenwood, B. M., E. M. Herrick, and E. J. Holborow. 1970. Speckled antinuclear factor in African sera. *Clin. Exp. Immunol.* 7: 75–83.
39. Mibei, E. K., A. S. Orago, and J. A. Stoute. 2005. Immune complex levels in children with severe *Plasmodium falciparum* malaria. *Am. J. Trop. Med. Hyg.* 72: 593–599.
40. Dalldorf, G., C. A. Linsell, F. E. Barnhart, and R. Martyn. 1964. An epidemiologic approach to the lymphomas of African children and Burkitt's sacroma of the jaws. *Perspect. Biol. Med.* 36: 435–449.
41. De Cock, K. M., A. N. Hodgen, R. A. Jupp, B. Slavin, T. K. Siongok, P. H. Rees, and S. B. Lucas. 1986. Immunoglobin M and malarial antibody levels in hyperreactive malarial splenomegaly. *J. Trop. Med. Hyg.* 89: 119–121.
42. Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc. Natl. Acad. Sci. USA* 99: 5545–5550.
43. Schutyser, E., A. Richmond, and J. Van Damme. 2005. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J. Leukocyte Biol.* 78: 14–26.
44. van der Voort, R., A. W. van Lieshout, L. W. Toonen, A. W. Sloetjes, W. B. van den Berg, C. G. Figdor, T. R. Radstake, and G. J. Adema. 2005. Elevated CXCL16 expression by synovial macrophages recruits memory T cells into rheumatoid joints. *Arthritis Rheum.* 52: 1381–1391.
45. Konig, A., V. Krenn, A. Toksoy, N. Gerhard, and R. Gillitzer. 2000. Mig, GRO α and RANTES messenger RNA expression in lining layer, infiltrates and different leucocyte populations of synovial tissue from patients with rheumatoid arthritis, psoriatic arthritis and osteoarthritis. *Virchows Arch.* 436: 449–458.
46. Nakayama, T., K. Hieshima, D. Izawa, Y. Tatsumi, A. Kanamaru, and O. Yoshie. 2003. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *J. Immunol.* 170: 1136–1140.
47. Tsubaki, T., S. Takegawa, H. Hanamoto, N. Arita, J. Kamogawa, H. Yamamoto, N. Takubo, S. Nakata, K. Yamada, S. Yamamoto, et al. 2005. Accumulation of plasma cells expressing CXCR3 in the synovial sublining regions of early rheumatoid arthritis in association with production of Mig/CXCL9 by synovial fibroblasts. *Clin. Exp. Immunol.* 141: 363–371.
48. Mackay, F., and J. L. Browning. 2002. BAFF: a fundamental survival factor for B cells. *Nat. Rev. Immunol.* 2: 465–475.
49. Dinarello, C. A. 2004. Therapeutic strategies to reduce IL-1 activity in treating local and systemic inflammation. *Curr. Opin. Pharmacol.* 4: 378–385.
50. McInnes, I. B., F. Y. Liew, and J. A. Gracie. 2005. Interleukin-18: a therapeutic target in rheumatoid arthritis? *Arthritis Res. Ther.* 7: 38–41.
51. Ngo, V. N., H. Korner, M. D. Gunn, K. N. Schmidt, D. S. Riminton, M. D. Cooper, J. L. Browning, J. D. Sedgwick, and J. G. Cyster. 1999. Lymphotoxin α/β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189: 403–412.
52. Ishikawa, S., S. Nagai, T. Sato, K. Akadegawa, H. Yoneyama, Y. Y. Zhang, N. Onai, and K. Matsushima. 2002. Increased circulating $CD11b^+CD11c^+$ dendritic cells (DC) in aged BWF1 mice which can be matured by TNF-α into BLC/CXCL13-producing DC. *Eur. J. Immunol.* 32: 1881–1887.
53. Peng, S. L. 2006. The T-box transcription factor T-bet in immunity and autoimmunity. *Cell. Mol. Immunol.* 3: 87–95.
54. Nemeth, E., S. Rivera, V. Gabayan, C. Keller, S. Taudorf, B. K. Pedersen, and T. Ganz. 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J. Clin. Invest.* 113: 1271–1276.

MAJOR ARTICLE

Effects of Sex, Parity, and Sequence Variation on Seroreactivity to Candidate Pregnancy Malaria Vaccine Antigens

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Background. *Plasmodium falciparum*–infected erythrocytes adhere to chondroitin sulfate A (CSA) to sequester in the human placenta, and pregnancy malaria (PM) is associated with the development of disease in and the death of both mother and child. A PM vaccine appears to be feasible, because women become protected as they develop antibodies against placental infected erythrocytes (IEs). Two IE surface molecules, VAR1CSA and VAR2CSA, bind CSA in vitro and are potential vaccine candidates.

Methods. We expressed all domains of VAR1CSA and VAR2CSA as mammalian cell surface proteins, using a novel approach that allows rapid purification, immobilization, and quantification of target antigen. For serum samples from East Africa, we measured reactivity to all domains, and we examined the effects of host sex and parity, as well as the effects of parasite antigenic variation.

Results. Serum samples obtained from multigravid women had a higher reactivity to all VAR2CSA domains than did those obtained from primigravid women or from men. Conversely, serum samples obtained from men had consistently higher reactivity to VAR1CSA domains than did those obtained from gravid women. Seroreactivity was strongly influenced by antigenic variation of VAR2CSA Duffy binding–like domains.

Conclusions. Women acquire antibodies to VAR2CSA over successive pregnancies, but they lose reactivity to VAR1CSA. Serum reactivity to VAR2CSA is variant specific, and future studies should examine the degree to which functional antibodies, such as binding-inhibition antibodies, are variant specific.

Plasmodium falciparum parasites sequester in the human placenta [1], and pregnancy malaria (PM) is associated with the development of disease in and the death of both mother and child [2–5]. Previous studies identified chondroitin sulfate A (CSA) as a major receptor molecule for sequestration of infected erythrocytes (IEs) in the placenta [6]. Malaria parasites variably express antigens on the IE surface that bind a variety of endothelial receptors [7, 8], including CSA. PfEMP1 is a variant surface antigen family encoded by ~60 *var* genes per malaria parasite genome [9], and these proteins have been implicated in a number of binding interactions. The sequences of *var* genes vary substantially within and between genomes. PfEMP1 forms are expressed in a mutually exclusive manner [10], creating extensive antigenic variation and the potential for multiple adhesion profiles. This variation is a major obstacle to the development of a PfEMP1-based antimalarial vaccine.

Resistance to PM increases over successive pregnancies [3] as women acquire antibodies against placental parasites. Serum samples obtained from immune multigravid women, but not those from men, can inhibit binding of placental IEs to CSA [11], even IEs collected in distant geographic regions. This serum activity is related to protection from infection and disease during pregnancy [12, 13]. Two PfEMP1 molecules, VAR1CSA and VAR2CSA, have been implicated in PM and are potential vaccine candidates (reviewed in [14]). Both are large molecules of >350 kDa with 7 and 6 distinct

Received 22 November 2006; accepted 26 January 2007; electronically published 23 May 2007.

Potential conflicts of interest: none reported.

Financial support: The Bill & Melinda Gates Foundation (grant 29202); National Institutes of Health (grant R01AI52059 to P.E.D.).

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The Journal of Infectious Diseases 2007; 196:155–64

0022-1899/2007/19601-0023$15.00
DOI: 10.1086/518513

Duffy binding–like (DBL) domains, respectively, and each is extensively cross-linked by disulfide bonds.

To study the role of these molecules in protective immunity, we expressed all domains of VAR1CSA and VAR2CSA on the surface of mammalian cells as green fluorescent protein (GFP) fusion proteins, by use of a novel vector that allowed rapid purification, immobilization, and quantification of antigen. We prepared arrays of individual VAR1CSA and VAR2CSA domains from laboratory strains and field isolates, and we tested their immunoreactivity by use of serum samples obtained from East African donors, to determine the effects of host sex and parity, as well as the effects of parasite antigenic variation, on antibody recognition.

MATERIALS AND METHODS

Vector for the expression of malaria antigens on the surface of mammalian cells. The DNA sequence encoding enhanced GFP (EGFP) was excised from pEGFP-N1 (Clontech) by means of *XhoI*/*NotI* digestion. The sequence encoding the transmembrane and cytoplasmic (TMC) domains of the rat surface receptor megalin [15] was amplified by use of reverse-transcription polymerase chain reaction (PCR) performed using forward and reverse primers with *Eco*RI and *Xho*I sites at their 5′ ends, respectively (forward primer: 5′-TTTGAATTCCTCCAGGGACGACAATGGCTGTT-3′; reverse primer: 5′-TTTCTCGAGTACGTCGGATCTTCTTTAACGAG-3′). The sequence then was digested with *Eco*RI and *Xho*I. Plasmid vector pSecTag2C (Invitrogen) was digested with *Bam*HI and *Eco*RI and then was ligated to a double-stranded (ds) oligonucleotide adaptor (AdEx) with a multicloning site created by annealing 2 single-stranded (ss) oligonucleotides: 5′-GATCCTTAAGTCCGGAGGCGCCTCTAGACTTAACGG-3′ and 5′-AATTCCGTTAACTCTAGAGGCGCCTCCGGACTTAAG-3′. The resulting vector was digested with *Eco*RI and *Xho*I and then was ligated to the megalin TMC fragment described above. This construct, in turn, was digested with *Xho*I and *Bsp*120I and was ligated to the EGFP fragment. The resulting vector was digested with *Xho*I and *Age*I to remove double-digestion sites, and it then was ligated to a ds oligonucleotide adaptor (created by annealing the following 2 ss oligonucleotides: 5′-TCGAGCTGAAGCTTCGAATCCTGCAGTCGACGGTACCGCGGGCCCGGGACCCA-3′ and 5′-CCGGTGGGTCCCGGGCCCGCGGTACCGTCGACTGCAGGATTCGAAGCTTCAGC-3′) that introduced point mutations to eliminate unwanted restriction sites. The resulting vector, known as "pAdEx," was used to clone and express the *P. falciparum* antigens described in the present study (figure 1). The integrity of the construct was verified by restriction digestion and sequencing.

Cloning malaria antigen genes into the pAdEx vector. DNA encoding each antigen was amplified by PCR from strain FCR3 and strain 3D7 *P. falciparum* genomic DNA or from cloned placental parasite sample 661 cDNA (see below), by use of PCR performed using primer pairs with appropriate restriction enzyme sites (table 1). After PCR was performed, amplified DNA fragments and the pAdEx vector were digested, ligated, and cloned. The integrity of each construct was verified by sequencing.

Cloning and sequencing of* var2csa *from placental parasite sample 661. Clinical placental parasite sample 661 was a placental intervillous blood sample obtained, after delivery, from a woman at Muheza Designated District Hospital (Muheza, Tanzania) who was participating in the Mother-Offspring Malaria Studies (MOMS) Project (described in [16]). Parasite samples were stored in RNALater (Ambion) at −20°C. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Purified RNA was treated with DNA-free reagent (Ambion) to remove genomic DNA. RNA was then reverse-transcribed using Superscript III and random hexamers (Invitrogen) for 2 h at 42°C. DBL6 forward primer 5′-AAGAACATTGTTCTAAATGTC-3′ and reverse primer 5′-TGTAAATATTGTTCAATAAAATCC-3′ were designed by aligning PFL0030c sequences from strains 3D7 and ITG (GenBank accession no. AY372123) to identify conserved sequences that flank the DBL6 domain. The PCR product was cloned into pCR2.1 vector by use of the TOPO TA Cloning System (Invitrogen) and was sequenced in both directions.

Preparation of quantitative protein arrays with malaria antigens. COS-7 cells (50%–70% confluent) were transfected with various constructs by use of Fugene transfection reagent (Roche) according to the manufacturer's protocol. Cells from

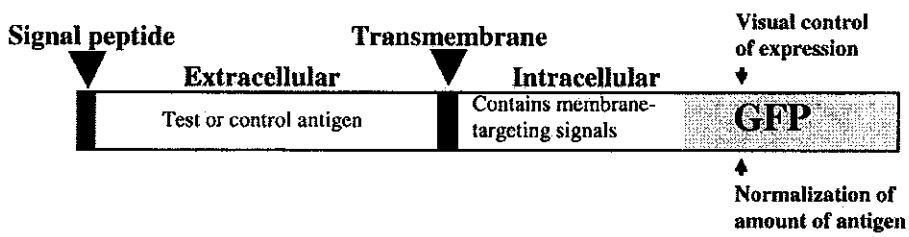

Figure 1. Hybrid protein for expression of *Plasmodium falciparum* antigens on the surface of mammalian cells. GFP, green fluorescent protein.

Table 1. Polymerase chain reaction primers for the amplification of antigen domains.

Domain (nucleotide positions)[a]	Forward primer[b]	Reverse primer[b]
VAR1CSA		
DBL1α-CIDR (271–2280)	CCCGGATCCAGGATCATAAGGAACATACTAATTTACGG	CCCGAATTCCATTTTTAGTGGGTTGCGTGCCTCCACG
DBL2β (2440–3402)	CCCCCTTAAGTCTAATCGTAATCTTGGTTTTTCAAATG	CCCGAATTCCAGACATTTGTGCTTGTTCATGTAATTC
DBL3γ (3802–4698)	TTCGGATCCTTAAAGAAAACGATGGAAAGAAAC	TTTGAATTCCATAGTCTGTAACCATTACACCAATG
DBL4ε (4855–5805)	CCCGGATCCAGGAAAATGACGACAAATATACTAACATT	CCCGAATTCCCTCGGAATATATTTTGTCTTTATTCTC
DBL5γ (5968–7146)	CCCGGATCCAGGACGATGAACCAAAAGAAGTTGAAGG	CCCGAATTCCATCCTTATACTTTTTGCCATCTTTATC
DBL6β (7594–8436)	CCCGGATCCAGGATAAATATATAGGAAGAAGAAACCC	CCCGAATTCCAGATTTCCATTTAAGAACAAAATTTTT
DBL7ε (8761–9540)	CCCGGATCCAGAAGGAATTACAAACTTTTACCTTCTG	CCCGAATTCCTTTATTGTCTATATTACCTGAAGATTG
VAR2CSA		
DBL1X (1–1347)	CCCCTCCGGAATGGATAAATCAAGTATTGCTAAC	CCCGAATTCCGATACATGTTTTATTCGACGACGG
DBL2X (1534–2586)	CCCCTCCGGATCTAGTTCTAATGGTAGTTGTAATAAC	CCCGAATTCCATTTGTAGTACTACTTGGGCCACAAT
DBL3X (3580–4557)	CCCGGATCCAGAAGGAAAATGAAAGTACCAATAATAAAA	CCCGAATTCCATCACTCGCAGATTTTCCTACATATTTA
DBL4ε (4708–5643)	CCCGGATCCAGGAGAAAAAAAATAATAAATCTCTTTG	CCCGAATTCCAGGTTCCATAATCATTGAATAATCTTT
DBL5ε (5944–7008)	CCCGGATCCAGTTAGATAGATGTTTTGACGACAAG	CCCGAATTCCTTTATTACAAATATAATCATTACC
DBL6ε (6973–7761)	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCTTTTTCTGCTTTGGTTTCTTTATAATTC
AMA-1 (70–1629)	CCCGGATCCAGGGACAGAATTATTGGGAACATCC	CCCTCTAGAATCATAAGTTGGTTTATGTTCAGG
MSP-1 19-kDa CTD (4588–5160)	CCCGGATCCAGATTGTTGAAAAAGATGAAGCACATG	CCCGAATTCCAATGAAACTGTATAATATTAACATG
661-VAR2CSA-DBL6ε	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCATTACCATTTTGGTTTTTAAATTTAGC

NOTE. AMA-1, apical membrane antigen–1; CIDR, cysteine-rich interdomain region; CTD, C-terminal domain; DBL, Duffy binding–like domain; MSP-1, merozoite surface protein–1.

[a] In the sequences of *var1csa* (FCR3 strain; GenBank accession no. AJ133811), *var2csa* (3D7 strain; PlasmoDB accession no. PFL0030c), *ama1* (3D7 strain; PlasmoDB accession no. PF11_0344), and *msp1* (3D7 strain; PlasmoDB accession no. PFI1475w).

[b] Restriction enzyme sites are underlined.

each 150-mm^2 flask were lysed 48 h after transfection (transfection efficiency, >80%) with 5 mL of CellLytic reagent (Sigma). Recombinant products were confirmed on Western blots with anti-GFP monoclonal antibody (MAb) (1:500 dilution; Clontech), followed by horseradish peroxidase (HRP)–conjugated anti–mouse IgG (1:1000 dilution; Sigma). Concentrations of fusion proteins (expressed in relative fluorescence units) were measured using GFP fluorescence with the use of the Fluoroskan Ascent FL fluorometer/luminometer (Thermo Labsystems), and they then were equalized by dilution with lysate of nontransfected cells (lysate K). A total of 100 μL of diluted lysate was added to each well of 384-well white plates coated with anti-GFP antibody (Pierce), and plates were incubated at 4°C overnight. Undiluted lysate K was used as a control for nonspecific background fluorescence and chemiluminescence. Lysate prepared from cells transfected with control construct (pAdEx vector alone without malaria antigen fusion partner) was used as a negative control in each assay. After washing with washing buffer (PBS plus 0.05% Tween-20), plates were ready for immunoprofiling experiments involving serum samples from humans.

Validation of expressed merozoite surface protein–1 (MSP-1) antigen by structure-sensitive monoclonal antibody. Recombinant MSP-1_{19} or control construct product AdEx was immobilized in anti-GFP plates as described above and then was incubated with mouse MAb 12.10 (1:5000 dilution), which is reactive only to the properly folded structure of MSP-1 [17] (provided by Dr. J. A. Lyon, Walter Reed Army Institute of Research), followed by HRP-conjugated anti–mouse IgG (1:1000 dilution; Sigma). Reactivity signals were obtained (expressed in relative luminescence units) by use of 100 μL of ECL chemiluminescence substrate (Amersham Biosciences) per well and a Fluoroskan luminometer.

Serum samples. The human serum samples used in these studies were collected from East African donors, under protocols approved by relevant ethics review committees. The study participants, who provided written, informed consent before donating samples, included adult men and multigravid women from Kenya [18, 19], as well as multigravid and primigravid women from Tanzania [20]. In brief, 18- to 45-year-old multigravid women and 18- to 50-year-old men (median age, 28 and 29 years, respectively; $P = .62$) from Kenya, as well as 18- to 45-year-old gravid women from Tanzania, were included in the study. Serum samples obtained from pregnant women were collected at the time of delivery and were tested individually. The number of serum samples used in each experiment is indicated in the corresponding figure legends. Serum samples obtained from 10 randomly selected nonimmune donors in the

United States were separated from whole blood obtained from commercial sources (Valley Biomedical) and were used in a pool as a negative control.

Immunoprofiling study of malaria antigens. All serum samples were preincubated at 4°C for at least 24 h with an equal volume of 10 mg/mL goat IgG, to eliminate nonspecific reactivity against goat anti–GFP IgG bound to the wells of 384-well plates. The preincubated serum samples were further diluted 1:100 with Superblock (Pierce) and were incubated with the antigen array for 2 h at room temperature. After 3 washes with washing buffer, plates were incubated with donkey anti-human IgG (H+L) affinity-purified antibody conjugated to HRP (Jackson Immunoresearch) diluted 1:1000 in Superblock. After 1 h at room temperature, the wells were washed; 100 μL of ECL chemiluminescence substrate (Amersham Biosciences) were then added per well, and chemiluminescence and fluorescence signals were measured. The use of the chemiluminescence substrate does not affect the fluorescence measurement.

Chemiluminescence signal reflects immune reactivity, and fluorescence signal reflects the amount of immobilized antigen–GFP fusion proteins. Fluorescence signal was corrected by subtraction of background values measured in lysate K wells, and then the immunoreactivity signal (chemiluminescence) was normalized to the amount of immobilized antigen (fluorescence) in each well. Average reactivity was calculated for duplicate wells, and a final specific immunoreactivity (expressed as arbitrary units [AUs]) was calculated by subtracting the control value (defined as either the average reactivity of the same serum sample to control construct +3 SD or the reactivity of pooled nonimmune serum samples to the same antigen +3 SD, whichever was greater). Correlations were analyzed using Spearman's rank test. Differences between group reactivities were tested for significance by use of the Mann-Whitney *U* test. $P < .05$ was considered to be statistically significant. GraphPad Prizm software was used for all statistical analyses.

RESULTS AND DISCUSSION

Features and performance of quantitative protein arrays. Heterologous expression of malaria surface antigens is known to be difficult, in part because of their high AT content (up to 80%) and their highly conformational cysteine-rich structure. An expression system that provides a transmembrane protein trafficking pathway and cell-surface presentation may significantly improve the cotranslational folding of PfEMP1 surface molecules, in which each domain contains 6–9 disulfide bonds. We engineered a pAdEx vector encoding a hybrid receptor with a signal peptide (from the immunoglobulin κ chain), an extracellular domain, and individual transmembrane and cytoplasmic domains (both from the single-spanning transmembrane receptor megalin) (figure 1). The cytoplasmic domain has signals that direct this protein to the plasma membrane surface. In addition, the GFP-reporter protein is fused to the cytoplasmic domain and reports protein expression levels, which can be quantified. The multicloning site allows simple and rapid preparation of different constructs that express *Plasmodium* antigen extracellular domains on the surface of mammalian cells.

Using this construct, we expressed several DBL domains from *var1csa* and *var2csa* genes, in addition to other *P. falciparum* antigens (the apical membrane antigen–1 [AMA-1] and MSP-1 19-kDa carboxy-terminal fragment), as GFP fusion proteins (figure 2). All antigens were successfully expressed using the native malaria coding sequence. Cysteine-rich interdomain region (CIDR)–α domains always follow DBL-α domains, and they may act as a single functional domain [9]; therefore,

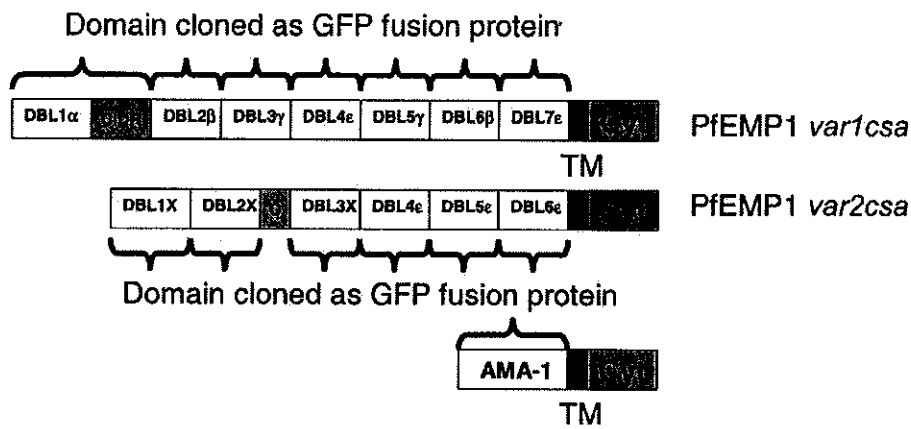

Figure 2. *Plasmodium falciparum* protein domains expressed and used for seroreactivity studies. Indicated domains were expressed as green fluorescent protein (GFP) fusion proteins in COS-7 cells and were immobilized individually for antigen arrays. As a positive control, apical membrane antigen–1 (AMA-1)–GFP fusion protein was used. Cyt, cytoplasmic domain; DBL, Duffy binding–like domain; TM, transmembrane domain.

var1csa DBL1-α domain was expressed together with CIDR1-α domain. For negative control wells, we used a GFP fusion protein (AdEx) containing an irrelevant extracellular domain of 37 aa that resulted from the translation of the multicloning site in the pAdEx DNA construct.

The integrity of fusion proteins was tested by Western blot analysis with anti-GFP antibodies (figure 3*A*). Recombinant proteins demonstrated the expected molecular weight and produced green fluorescence in cells as well as in cell lysates. Fluorescence was preserved after immobilization of fusion proteins in 384-well plates. GFP fluorescence has been shown to be a good indicator of properly folded membrane proteins when GFP is fused to the cytoplasmic tail [21]. We also tested the reactivity of the disulfide-rich MSP-1 19-kDa fusion protein by use of conformation-dependent MAb 12.10 [17], which readily recognized the antigen (figure 3*B*), thereby confirming correct folding.

Malaria antigens were organized into arrays by use of a single-step procedure performed in 384-well plates. The GFP fusion partner has a number of advantages. First, the tag can be used for immobilization and purification of antigens in a single simple step. Second, the GFP allows the amount of antigen in each lysate to be measured and equalized, thereby reducing variance. Third, the immunoreactivity of serum samples (measured by chemiluminescence) can be normalized to the amount of antigen (measured simultaneously by GFP fluorescence) in each well, which further reduces variance.

Seroreactivity to irrelevant antigens. As was observed in earlier studies [22, 23], we found that serum samples obtained from immune individuals in malaria-endemic regions frequently react to completely irrelevant proteins (data not shown), and this nonspecific reactivity corresponds to an elevated reactivity to malaria antigens. In contrast, serum samples obtained from nonimmune individuals (NISS) living in areas of nonendemicity have low nonspecific reactivity. For this reason, NISS control may not be adequate to demonstrate specific reactivity of serum samples tested in seroepidemiologic studies of malaria, because this approach may falsely identify serum samples with high levels of nonspecific reactivity as having a positive result. The use of the control construct provides the means to quantify and, therefore, correct for nonspecific reactivity of each construct in each serum sample.

Seroreactivity to VAR1CSA and VAR2CSA associated with a dichotomous pattern related to sex. We measured the seroreactivity of East African and nonimmune individuals to domains of VAR1CSA and VAR2CSA expressed as GFP fusion proteins. AMA-1 was used as a positive control because it is known to react strongly to the majority of serum samples obtained from individuals in malaria-endemic regions [24]. As expected, serum samples obtained from immune individuals uniformly showed high levels of reactivity to relatively conserved AMA-1, and seroreactivity did not differ between men and multigravid women (inset in figure 4*A*) (median for 44 serum samples obtained from men, 5289 AUs; median for 52

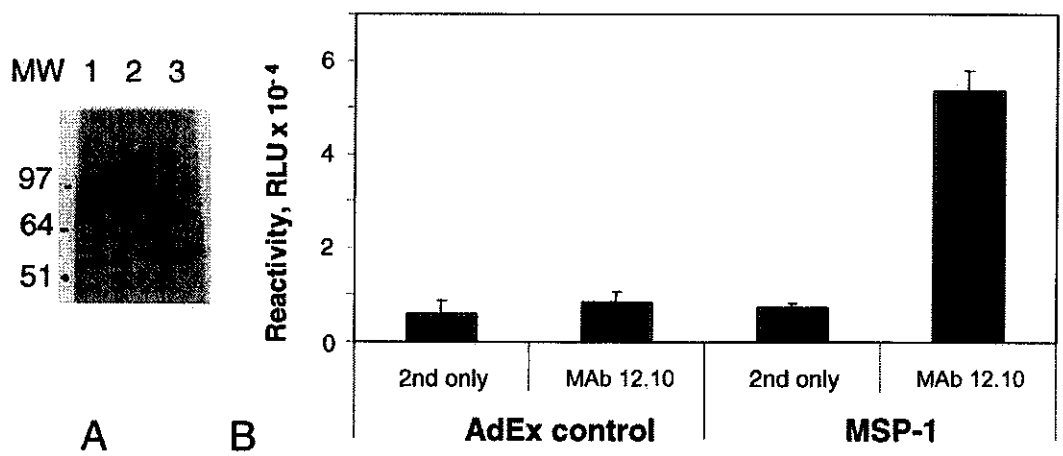

Figure 3. Characterization of malaria antigens cloned as green fluorescent protein (GFP) fusion proteins in the pAdEx vector and expressed in COS-7 cells. *A*, Western blot of expressed antigens with monoclonal anti-GFP antibody. Lane 1, Duffy binding–like (DBL) 3γ region from VAR1CSA (predicted molecular weight [MW], 85 kDa); lane 2, control construct minimegalin with extracellular domain containing the first ligand-binding domain of rat receptor megalin (nt 1–1882) [15] (predicted MW, 106 kDa); and lane 3, merozoite surface protein–1 (MSP-1) 19-kDa fragment (predicted MW, 70 kDa). *B*, Interaction of structure-sensitive anti–MSP-1 monoclonal antibody (MAb) 12.10 [17] with MSP-1 fusion protein. The control protein expressed from vector without insert (AdEx control) or MSP-1 recombinant protein was immobilized in the wells of anti-GFP plates and was tested for reactivity with monoclonal antibody (MAb) 12.10 followed by secondary anti–mouse horseradish peroxidase (HRP)–conjugated antibody (MAb 12.10) or with secondary antibody only (2nd only). Signals were measured using chemiluminescent substrate. Bars denote the average of 3 measurements, and error bars denote SEs. RLU, relative luminescence units.

serum samples obtained from multigravid women, 5872 AUs; $P = .46$).

Immune responses to PfEMP1 domains were substantially lower and more variable (figure 4) than were AMA-1 responses. Two VAR1CSA domains (DBL6β and DBL7ε) and 1 VAR2CSA domain (DBL2X) were found to be nonreactive or minimally reactive in our screening tests. Nonreactivity of VAR2CSA DBL2X was likely the result of rapid degradation of this fusion protein during and after cell lysate preparation, as detected by Western blot analysis (data not shown). The reason for nonreactivity of VAR1CSA DBL6 and DBL7 is not clear, because the proteins were stable. The results suggest that host immunoreactivity is weak against these domains, but we cannot exclude the possibility that the proteins were incorrectly folded in a way that disrupted or masked structural epitopes.

The variable response to VAR1CSA and VAR2CSA was related to the sex of the serum donors. Consistent with the findings of earlier studies from West Africa [25–27], the reactivity of all VAR2CSA domains (other than DBL-2X) was significantly higher with serum samples from multigravid women than with serum samples from men (figure 4). Of the 54 serum samples that were obtained from Kenyan multigravid women and were tested in this experiment, 10 were obtained from women with PM. Antibody levels were not significantly different (data not shown) in women with PM versus those without PM, possibly reflecting that the duration of infection is brief in the multigravid women [3, 28, 29] or that antibody levels may be maximal in this parity group by the time of delivery. Previous studies in West Africa that examined 3 DBL domains (DBL1, DBL5, and DBL6) of VAR2CSA expressed in baculovirus [25, 26] found that seroreactivity to domains 5 and 6, but not to domain 1, was significantly higher in multigravid women than in men. The increased reactivity against all VAR2CSA domains noted for serum samples obtained from multigravid women in East Africa supports the idea that this PfEMP1 molecule is preferentially expressed by PM parasites and that women acquire antibodies against this protein as they become protected. Reactivity to the DBL1X domain was significantly correlated with reactivity against 3 other domains (Spearman correlation for DBL3, $r = 0.29$ [$P = .04$]; for DBL5, $r = 0.41$ [$P = .003$];

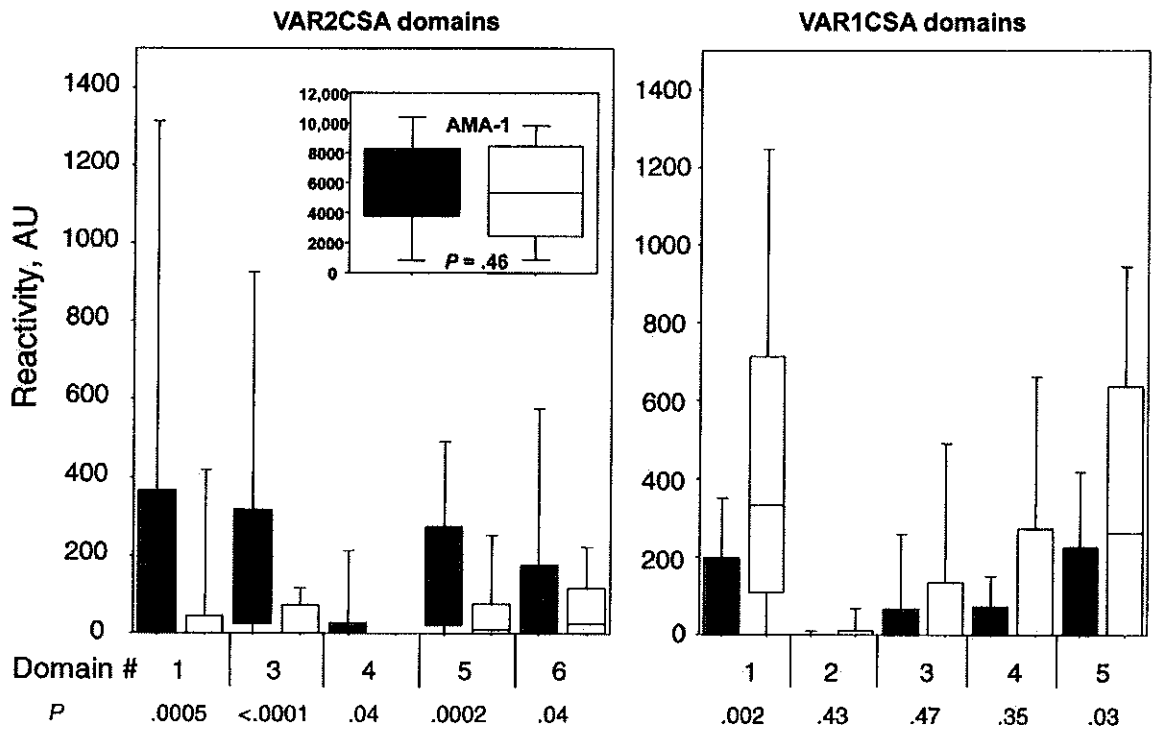

Figure 4. Preferential reaction of serum samples from multigravid women to VAR2CSA domains and preferential reaction of serum samples obtained from men to VAR1CSA domains. Seroreactivity to VAR2CSA and VAR1CSA domains (after subtraction of the control value [see Materials and Methods]) is indicated according to donor group. White bars denote serum samples obtained from men, and gray bars denote serum samples obtained from multigravid women. AU, arbitrary units. *P* values are the results of a 2-tailed Mann-Whitney *U* test (for 52 serum samples obtained from multigravid women and 44 serum samples obtained from men *[left]* and for 32 serum samples obtained from multigravid women and 32 serum samples obtained from men *[right]*). The top of the box denotes the 75th percentile, the bottom of the box denotes the 25th percentile, and the line through the middle of the box denotes the 50th percentile (i.e., the median). The whiskers denote the 10th and 90th percentiles. The inset shows the reactivity of apical membrane antigen–1 (AMA-1) for both groups.

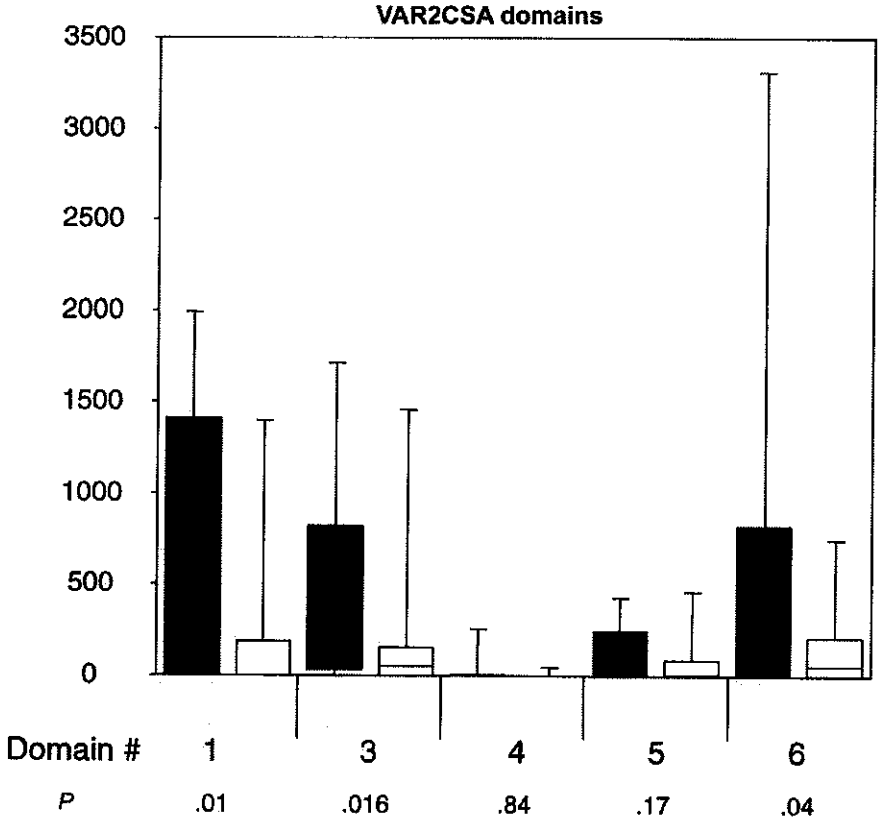

Figure 5. Increases in serum reactivity to VAR2CSA domains with gravidity. Seroreactivity to individual VAR2CSA domains is stratified by gravidity. White bars denote serum samples obtained from primigravid women, and gray bars denote serum samples obtained from multigravid women. AU, arbitrary units. *P* values are results of a 2-tailed Mann-Whitney *U* test ($n = 32$ for each group). The top of the box denotes the 75th percentile, the bottom of the box denotes the 25th percentile, and the line through the middle of the box denotes the 50th percentile (i.e., the median). The whiskers denote the 10th and 90th percentiles.

and for DBL6, $r = 0.48$ [$P = .0003$]) but not against AMA-1 antigen ($r = 0.07$; $P = .6$), suggesting that immunity to different VAR2CSA domains is acquired concordantly.

The pattern of reactivity to VAR1CSA versus VAR2CSA domains diverged markedly and was consistent against all tested domains (figure 4). Serum samples obtained from multigravid women reacted more strongly to VAR2CSA domains, whereas serum samples obtained from men reacted more strongly to VAR1CSA domains. The increased antibody levels noted in men versus those noted in multigravid women were statistically significant for 2 VAR1CSA domains (DBL1α and DBL5γ). Men and multigravid women had similar reactivity to AMA-1 (see above) and MSP-1 (data not shown), indicating that multigravid women specifically lose reactivity to VAR1CSA. Our studies of AMA-1 and MSP-1_{19} are similar to numerous earlier studies, which found that seroreactivity to various non-PfEMP1 malaria antigens did not vary with the pregnancy status or parity of the sample donors [30].

The dichotomous pattern of reactivity of men and multigravid women may be explained by mutually exclusive expression of *var* genes in *P. falciparum* [10]. PM is caused by CSA-binding parasites [6] that preferentially express *var2csa* [31], and peripheral parasites in pregnant women have features similar to those of placental parasites [16, 32]. Thus, the up-regulation of *var2csa* in placental parasites may be accompanied by a down-regulation of other commonly expressed *var* genes, such as *varIcsa*. Antibodies against VAR1CSA domains may therefore diminish in pregnant women, who would receive antigenic stimulation by VAR2CSA but not VAR1CSA during episodes of PM.

Gravidity-related increases in seroreactivity to VAR2CSA. We compared immunoreactivity to VAR2CSA domains in serum samples obtained from multigravid women (all 32 of whom did not have PM) and those obtained from primigravid women (8 of whom had PM and 24 of whom did not) in Tanzania. As was observed elsewhere [25, 26] (see below), VAR2CSA seroreactivity increased with the number of pregnancies (figure 5) and, consequently, with protection status.

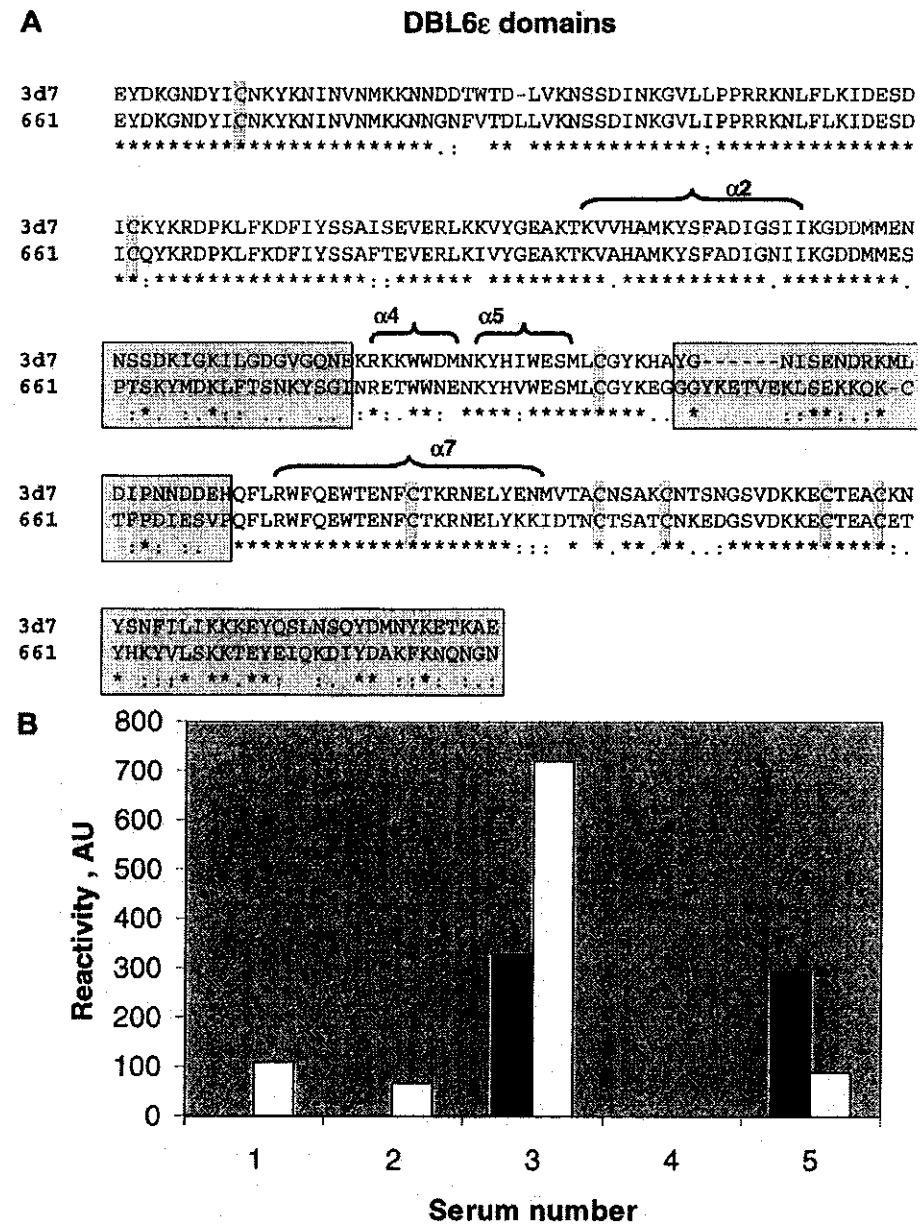

Figure 6. Antigenic variation and seroreactivity of VAR2CSA Duffy binding–like (DBL) 6ε domain. *A*, Comparison of strain 3D7 and placental parasite sample 661 DBL6ε domain sequences. Sequence alignment was performed using Clustal W at the GenomeNet Web site (available at: http://align.genome.jp). Stars denote conserved residues, and colons and dots denote more- and less-conservative substitutions, respectively. α2, α4, α5, and α7 are helical regions identifiable according to information found in [34]. Blue boxes denote regions of low homology. Yellow boxes denote cysteines conserved between these 2 variants. *B*, Reactivity of the 3D7 strain and the placental parasite sample 661 DBL6ε domains with serum samples obtained from 5 multigravid women. The 5 samples were randomly selected from the set of serum samples used in previously described experiments. Black bars denote 3D7 DBL6ε; white bars, 661 DBL6ε.

Differences in seroreactivity between groups of differing gravidities were statistically significant for 3 domains (DBL1, DBL3, and DBL6). These differences remained significant in analyses that included only serum samples from women without PM. In earlier studies, seroreactivity of the DBL5 domain [25] and 3 VAR2CSA domains (DBL1, DBL5 and DBL6) [26] correlated significantly with gravidity, but the levels of seroreactivity were not significantly different between groups of differing gravidities. Interestingly, antibody levels to VAR2CSA domains 1, 3, and 6 were significantly higher ($P < .05$, for all comparisons;

data not shown) among first-time Tanzanian mothers with PM versus those without PM in our study, suggesting specific responses to the antigen during PM. Separate studies will need to examine whether the antibodies produced by first-time mothers during malaria episodes have functional activity.

Similar studies were previously undertaken in West Africa with 2 VAR1CSA DBL domains (DBL1 and DBL2) expressed in *Escherichia coli* [33] and with varying numbers of VAR2CSA domains (2 [25], 3 [26], or 6 [27]) expressed in the baculovirus system. In those studies, for serum samples obtained from men and women, the reactivity against VAR1CSA domains did not differ significantly. Of note, *E. coli*–expressed DBL antigens may not recreate the extensive disulfide bonds and folds of the native protein [14], and expression of the DBL1 domain separate from the CIDR domain may disrupt a single functional domain and alter its conformation. In our studies, VAR1CSA domains were expressed on the surface of mammalian cells to better reproduce the native structure of these complex antigens, which may allow better discrimination of differences in seroreactivity. The earlier studies of VAR2CSA, which we discussed in detail above, generally observed a sex-specific and parity-specific pattern of reactivity, supporting the idea that VAR2CSA is preferentially expressed by placental parasites and is targeted by antibodies that correlate with immunity.

In our work, we expanded on these previous studies to incorporate all domains from each PfEMP1 protein, expressed each domain in a mammalian system to increase the probability of correct folding, and studied them together by use of serum samples from a distinct geographic region, East Africa. To our knowledge, these are the first studies to show a higher level of recognition of all immunoreactive VAR1CSA domains by serum samples obtained from men versus those obtained from multigravid women, and reactivity is highest against the first VAR1CSA domain (DBL1α plus CIDR1α). We also demonstrated that all immunoreactive VAR2CSA domains react most strongly to serum samples obtained from multigravid women, and we confirmed that this reactivity is parity specific.

Variant-specific reactivity to VAR2CSA. We compared the reactivity of immune serum samples to variant forms of domain DBL6ε representing laboratory isolate 3D7 and fresh placental parasite sample 661. These variant forms have a high level of homology throughout most of their sequence (figure 6*A*). Individual serum samples obtained from multigravid women varied substantially in their reactivity to variant forms of DBL6ε (figure 6*B*). Antigenic variation in this domain is limited to areas comprising ~30% of the domain sequence, primarily in the loops between helices α2 and α4, as well as those between helices α5 and α7 [34]. Because the remainder of the domain is largely conserved, and because the immune response against these 2 homologous domains is significantly different, we speculate that the immune response is predominantly directed toward regions of sequence variability, including the loops. This may also indicate that the most conserved parts of the domain are poorly immunogenic. We also saw a similar pattern of differential reactivity with VAR2CSA DBL1X domains (identity was ~80% between variants [data not shown]).

A previous study by Tuikue Ndam et al. [26] demonstrated no association between serum levels of anti-3D7 VAR2CSA antibodies and anti–CSA-binding antibodies in 4 of 6 placental isolates. This may have resulted from VAR2CSA sequence variation between placental samples, as the authors suggested, or it may indicate that functional antibodies are a minor subset of total antibodies. Our results regarding differential reactivity of laboratory isolate (3D7) versus placental parasite (sample 661) DBL domains do not confirm one or the other of these possibilities. If the former possibility is correct, then protective immunity in multigravid women may reflect the acquisition of antibodies against the VAR2CSA variants present in a community. A globally related pool of polymorphisms accounts for sequence variation in VAR2CSA [35], and, therefore, a limited number of variants may be adequate to elicit broadly reactive antibodies. Such a vaccine may be able to target only the loop regions, which could significantly simplify the task of developing a vaccine

Future studies will need to identify the malaria antigen, domain, or domain variant(s) and fragment(s) that are specifically targeted by protective antibodies, as well as those that elicit broadly reactive antibodies. This information could provide the basis for a PM vaccine.

Acknowledgments

We thank Vlad Malkov for fruitful discussion. J. Lyon provided antibody to MSP-1.

References

1. Walter PR, Garin Y, Blot P. Placental pathologic changes in malaria: a histologic and ultrastructural study. Am J Pathol **1982**; 109:330–42.
2. Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull World Health Organ **1983**; 61:1005–16.
3. McGregor IA. Thoughts on malaria in pregnancy with consideration of some factors which influence remedial strategies. Parassitologia **1987**; 29:153–63.
4. Murphy SC, Breman JG. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am J Trop Med Hyg **2001**; 64:57–67.
5. Guyatt HL, Snow RW. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. Clin Microbiol Rev **2004**; 17:760–9.
6. Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science **1996**; 272:1502–4.
7. Baruch DI, Ma XC, Singh HB, Bi X, Pasloske BL, Howard RJ. Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence. Blood **1997**; 90:3766–75.
8. Rowe JA, Moulds JM, Newbold CI, Miller LH. *P. falciparum* rosetting

mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. Nature **1997**; 388:292–5.
9. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **2002**; 419:498–511.
10. Scherf A, Hernandez-Rivas R, Buffet P, et al. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in *Plasmodium falciparum*. EMBO J **1998**; 17:5418–26.
11. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature **1998**; 395:851–2.
12. Duffy PE, Fried M. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. Infect Immun **2003**; 71: 6620–3.
13. Staalsoe T, Shulman CE, Bulmer JN, Kawuondo K, Marsh K, Hviid L. Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated *Plasmodium falciparum* malaria. Lancet **2004**; 363:283–9.
14. Rowe JA, Kyes SA. The role of *Plasmodium falciparum var* genes in malaria in pregnancy. Mol Microbiol **2004**; 53:1011–9.
15. Saito A, Pietromonaco S, Loo AK, Farquhar MG. Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. Proc Natl Acad Sci USA **1994**; 91:9725–9.
16. Fried M, Domingo GJ, Gowda CD, Mutabingwa TK, Duffy PE. *Plasmodium falciparum:* chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta. Exp Parasitol **2006**; 113:36–42.
17. Angov E, Aufiero BM, Turgeon AM, et al. Development and pre-clinical analysis of a *Plasmodium falciparum* merozoite surface protein-1(42) malaria vaccine. Mol Biochem Parasitol **2003**; 128:195–204.
18. Kurtis JD, Lanar DE, Opollo M, Duffy PE. Interleukin-10 responses to liver-stage antigen 1 predict human resistance to *Plasmodium falciparum*. Infect Immun **1999**; 67:3424–9.
19. Fried M, Muga RO, Misore AO, Duffy PE. Malaria elicits type 1 cytokines in the human placenta: IFN-γ and TNF-α associated with pregnancy outcomes. J Immunol **1998**; 160:2523–30.
20. Mutabingwa TK, Bolla MC, Li JL, et al. Maternal malaria and gravidity interact to modify infant susceptibility to malaria. PLoS Med **2005**; 2: e407.
21. Drew DE, von Heijne G, Nordlund P, de Gier JW. Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli*. FEBS Lett **2001**; 507:220–4.
22. Knobloch J, Schreiber M, Grokhovsky S, Scherf A. Specific and nonspecific immunodiagnostic properties of recombinant and synthetic *Plasmodium falciparum* antigens. Eur J Clin Microbiol **1987**; 6:547–51.
23. Fesel C, Goulart LF, Silva Neto A, et al. Increased polyclonal immunoglobulin reactivity toward human and bacterial proteins is associated with clinical protection in human *Plasmodium* infection. Malar J **2005**; 4:5.
24. Cortes A, Mellombo M, Masciantonio R, Murphy VJ, Reeder JC, Anders RF. Allele specificity of naturally acquired antibody responses against *Plasmodium falciparum* apical membrane antigen 1. Infect Immun **2005**; 73:422–30.
25. Salanti A, Dahlback M, Turner L, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med **2004**; 200: 1197–203.
26. Tuikue Ndam NG, Salanti A, Le-Hesran JY, et al. Dynamics of anti-VAR2CSA immunoglobulin G response in a cohort of Senegalese pregnant women. J Infect Dis **2006**; 193:713–20.
27. Barfod L, Nielsen MA, Turner L, et al. Baculovirus-expressed constructs induce immunoglobulin G that recognizes VAR2CSA on *Plasmodium falciparum*-infected erythrocytes. Infect Immun **2006**; 74:4357–60.
28. McGregor I. Malaria—recollections and observations. Trans R Soc Trop Med Hyg **1984**; 78:1–8.
29. Brabin B, Rogerson S. The epidemiology and outcomes of maternal malaria. In: Duffy PE, Fried M, eds. Malaria in pregnancy. New York, London: Taylor and Francis, **2001**:27–52.
30. Fievet N, Cot M, Chougnet C, et al. Malaria and pregnancy in Cameroonian primigravidae: humoral and cellular immune responses to *Plasmodium falciparum* blood-stage antigens. Am J Trop Med Hyg **1995**; 53:612–7.
31. Salanti A, Staalsoe T, Lavstsen T, et al. Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A–adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol Microbiol **2003**; 49:179–91.
32. Ofori MF, Staalsoe T, Bam V, et al. Expression of variant surface antigens by *Plasmodium falciparum* parasites in the peripheral blood of clinically immune pregnant women indicates ongoing placental infection. Infect Immun **2003**; 71:1584–6.
33. Jensen AT, Zornig HD, Buhmann C, et al. Lack of gender-specific antibody recognition of products from domains of a *var* gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. Infect Immun **2003**; 71:4193–6.
34. Singh SK, Hora R, Belrhali H, Chitnis CE, Sharma A. Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. Nature **2006**; 439:741–4.
35. Trimnell AR, Kraemer SM, Mukherjee S, et al. Global genetic diversity and evolution of var genes associated with placental and severe childhood malaria. Mol Biochem Parasitol **2006**; 148:169–80.

Available online at www.sciencedirect.com
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Molecular & Biochemical Parasitology 155 (2007) 57–65
MOLECULAR & BIOCHEMICAL PARASITOLOGY

The distinct proteome of placental malaria parasites

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Received 11 April 2007; received in revised form 22 May 2007; accepted 24 May 2007
Available online 29 May 2007

Abstract

Malaria proteins expressed on the surface of *Plasmodium falciparum* infected erythrocytes (IE) mediate adhesion and are targeted by protective immune responses. During pregnancy, IE sequester in the placenta. Placental IE bind to the molecule chondroitin sulfate A (CSA) and preferentially transcribe the gene that encodes VAR2CSA, a member of the PfEMP1 variant surface antigen family. Over successive pregnancies women develop specific immunity to CSA-binding IE and antibodies to VAR2CSA. We used tandem mass spectrometry together with accurate mass and time tag technology to study IE membrane fractions of placental parasites. VAR2CSA peptides were detected in placental IE and in IE from children, but the MC variant of VAR2CSA was specifically associated with placental IE. We identified six conserved hypothetical proteins with putative TM or signal peptides that were exclusively expressed by the placental IE, and 11 such proteins that were significantly more abundant in placental IE. One of these hypothetical proteins, PFI1785w, is a 42 kDa molecule detected by Western blot in parasites infecting pregnant women but not those infecting children.

Keywords: Placental malaria; *Plasmodium falciparum*; Membrane-associated proteins; Comparative proteomic

1. Introduction

Young children and pregnant women, particularly women pregnant for the first time, are highly susceptible to the malaria parasite *Plasmodium falciparum*. Disease and death are related to the ability of intraerythrocytic *P. falciparum* to bind endothelium and sequester in deep vascular beds. During blood stage development, *P. falciparum* parasites export proteins that mediate adhesion and sequestration to the surface of the infected erythrocyte (IE) [1]. In pregnant women, IE adhere to chondroitin sulfate A (CSA) and sequester in the placenta, often leading to placental inflammatory responses associated with maternal anemia and low birth weight [2].

Placental IE have a distinct adhesion profile, binding to CSA but not to other receptors like CD36 and ICAM1 that commonly support adhesion of other IE forms [3]. Specific immune responses to placental IE develop over successive pregnancies, and these are associated with reduced infection and improved pregnancy outcomes [4,5]. The IE surface antigens targeted by naturally acquired antibodies appear to have conserved features in CSA-binding parasites from around the globe [4], suggesting that they may be broadly effective as a vaccine. The variant antigen gene *var2csa* has been shown to be upregulated in CSA-binding and placental parasites, and to encode domains that bind to CSA *in vitro*, suggesting a role for the VAR2CSA protein in placental malaria [6–8].

Although significant effort is ongoing to understand the role of VAR2CSA in placental malaria, it is unknown whether other IE surface proteins may be differentially expressed by placental parasites. To date, several proteomic studies of *P. falciparum* parasites have been published, including global proteomic studies

Abbreviations: IE, infected erythrocyte; CSA, chondroitin sulfate A; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; TM, transmembrane; AMT, accurate mass and time

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doi:10.1016/j.molbiopara.2007.05.010

that compared protein profiles during different developmental stages of the parasite life cycle, as well as targeted studies to identify IE membrane proteins and other organelle-specific proteomes [9–12]. We recently used tandem mass spectrometry (MS/MS) to profile the PfEMP1 proteins (encoded by *var* genes) expressed by parasites collected from children versus pregnant women [13]. No studies have reported the total IE membrane proteome of fresh parasite samples, including fresh placental IE samples.

We now have used a label-free comparative proteome strategy to identify and relate IE surface protein differences between parasites collected from children versus those from pregnant women. This strategy incorporates the accurate mass and time (AMT) tag [14] approach for organism-wide comparative protein measurements, which has been previously applied to studies of the human blood plasma proteome [15] and studies of prokaryotic proteomes [16,17]. In this study, MS/MS was used to identify the IE surface proteome, and Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) was employed to confirm tandem mass spectrometry findings [14] and to compare protein abundance in the two types of samples by AMT tags [16]. The results suggest that the membrane proteome of placental IE includes a distinct repertoire of highly conserved proteins encoded by single-copy genes, in addition to PfEMP1 proteins.

2. Materials and methods

2.1. Parasite samples and binding assays

Parasite samples were collected from pregnant women and their children participating in the MOMS Project. MOMS Project is a longitudinal birth cohort study launched in 2002 in Muheza, Tanzania, an area of intense malaria transmission. Details of the project have been reported elsewhere [18]. The present study reports analyses of 18 freshly collected placental parasite samples and 21 parasite samples collected from infected children. Placental IE are typically mature parasite forms (called trophozoites and schizonts) sequestered in the intervillous spaces of the placenta, and for this study were obtained by mechanical grinding of the placenta. IE from infected children were obtained from peripheral blood, where they circulate as immature non-adherent parasite forms referred to as ring stage parasites. IE collected from children were therefore allowed to mature to the trophozoite/schizont stages by *in vitro* culture for 16–20 h [19]. Binding phenotypes of parasite samples were determined in a static binding assay that measures adhesion of mature stage IE to immobilized receptors, as previously described [3].

2.2. Preparation of samples for proteomics studies

Mature forms of the parasites were purified on Percoll gradients. Enriched samples contained more than 90% IE and <10% uninfected erythrocytes. Membrane proteins were enriched by sequential extraction with detergent [13]. Parasite samples were incubated in lysis buffer A (10 mM Tris–HCl pH 7.4, 5 mM EDTA, 1% Triton X-100) for 30 min at 4 °C, then the lysates were centrifuged for 20 min at 12,000 × *g* at 4 °C to pellet insoluble material. After removing the supernatants, pellets were extracted in lysis buffer B (10 mM Tris–HCl pH 7.4, 5 mM EDTA, 2% SDS, 6 M Urea) to solubilize membrane-associated proteins. As we have previously shown, the membrane-associated protein KAHRP is detected in the SDS soluble fraction but not in the Triton X-100 fraction, whereas the cytoplasmic protein BiP is present in the Triton X-100 fraction but is significantly reduced in SDS fraction [13].

To prepare trypsin digestion peptides, the membrane-associated proteins were reduced with 10 mM DTT for 1 h at 37 °C, then alkylated with 20 mM iodoacetamide for 1 h at 25 °C. The samples were diluted to 0.05% SDS with 25 mM NH_4CO_3, and trypsin was added to a final enzyme:substrate ratio of 1:50 (w/w). The samples were digested overnight at 37 °C. After trypsin digestion, peptides were desalted using HILIC (The Nest Group Inc.), according to the manufacturer's instructions.

2.3. LC–MS/MS analysis using ion trap

LC–MS/MS was performed using LCQ Deca XP and LTQ-MS ion trap mass spectrometers (ThermoFinnigan). A total of 5 μg of total peptide at 1 μg/μL (as determined by the BCA assay) were loaded onto the reversed-phase column using a two-mobile-phase solvent system consisting of 0.4% acetic acid in water (A) and 0.4% acetic acid in acetonitrile (B).

The mass spectrometer operated in a data-dependent MS/MS mode over the *m/z* range of 400–2000. For each cycle, the three or the five most abundant ions from each MS scan were selected for MS/MS analysis using LCQ Deca XP or LTQ-MS, respectively using 45% collision energy. Dynamic exclusion was used to exclude previously analyzed ions in a 1 min window.

The Sequest algorithm was used to match MS/MS spectra to peptides in the sequence database. The database included *P. falciparum* 3D7 genome sequence and other *Plasmodium* sequences submitted to NCBI, as well as the sequences from the non-redundant human database. Spectra/peptide matches were determined to be positive if the ΔC_n value was 0.1 or greater, and the X_{corr} was sufficiently high depending on the charge state of the peptide: for charge state +1, $X_{corr} \geq 1.5$ for full tryptic peptides or $X_{corr} \geq 3.1$ for partially cleaved peptides; for charge state +2, $X_{corr} \geq 1.9$ for full tryptic peptides or $X_{corr} \geq 3.8$ for partially cleaved peptides; for charge state +3, $X_{corr} \geq 2.9$ for full tryptic peptides or $X_{corr} \geq 4.5$ for partially cleaved peptides [20].

2.4. Accurate mass and time (AMT) tag approach using FTICR

To perform FTICR analysis, 5 μg of peptide mixtures were separated by HPLC using a reversed-phase capillary column (150 μm i.d. × 360 μm o.d., Polymico Technologies) and a two-mobile-phase solvent system consisting of 0.2% acetic acid and 0.05% trifluoroacetic acid (TFA) in water and 0.1% TFA in 90% acetonitrile/10% water. Peptides were identified by FTICR mass spectrometer, and the spectra were matched to potential mass

and time (PMT) tags previously identified by LC–MS/MS studies of the same sample to define AMT tags. The FTICR data were analyzed using software developed in-house (PNNL) that uses average peptide peak intensities to estimate protein abundance [14,21]. The data were normalized using a cluster analysis [22]. Hierarchical clustering and statistical analysis (Spearman rank correlation; Mann–Whitney *U* test) were performed using Acuity 4.0 (Axon). The average number of peptides in placental and children's parasite samples was 2774 and 4177, respectively.

2.5. Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed on placental and children's parasites to survey differential transcription of the genes corresponding to differentially expressed proteins. Total RNA was purified from mature IE samples (i.e., fresh placental IE samples or IE samples from children after overnight culture) using RNAwiz (Ambion). Reverse transcription was performed using oligo-dT_{20} primer and Superscript II enzyme (Invitrogen). Quantitative PCR on cDNA samples was performed using SYBR green master mix (Applied Biosystems) in an ABI Prism 7000 thermal cycler (Applied Biosystems). Primers were validated using both genomic DNA and a pool of cDNA, and demonstrated linear amplification over a range of template dilutions. Threshold cycles (C_T) were calculated and normalized ($\Delta\Delta C_T$ method) using C_T values for the *P. falciparum* housekeeping gene seryl-tRNA synthetase. The results were used to calculate fold-differences between placental and children's IEs, and the statistical significance of these differences was determined by Student's *t*-test using Statview software (SAS Inc.).

2.6. Protein expression and antibody production

PFI1785w was amplified from the cDNA of a placental parasite sample and cloned into pET28b vector (Novagen) for expression in *E. coli* cells. His-tagged recombinant PFI1785w (rPFI1785w) was purified on a nickel column (Novagen) according to the manufacturer's instructions. The protein was further purified by weak ion exchange HPLC then eluted in increasing salt concentrations (0.05–1.00 M NaCl in 20 mM Tris pH 7.4).

Specific antisera were obtained by immunizing Balb/c mice three times with 25 μg of rPFI1785w emulsified in TiterMax Gold adjuvant.

2.7. Western blot analysis

Ten micrograms of surface membrane fractions of placental IE and IE from children were loaded onto 4–12% NuPAGE gels (Invitrogen, CA). The gels were transferred to PVDF membrane (Invitrogen, CA), blocked with 5% BSA in PBS-0.05% Tween 20, then incubated with polyclonal mouse antisera to PFI1785w or monoclonal antibody to knob-associated histidine rich protein (KAHRP) (kindly provided by Dr. D.W. Taylor) [23] followed by incubation with anti-mouse antibodies conjugated to HRP (Sigma). Chromogenic CN/DAB kit (Pierce, IL) was used to visualize the antigens recognized by specific antibodies.

3. Results and discussion

3.1. Parasite proteins identified exclusively in placental parasites by LC–MS/MS

Our goal in this study was to identify the IE surface proteomes of parasites with distinct adhesion phenotypes. Placental parasites have a distinct binding phenotype, suggesting that specific proteins are uniquely or preferentially expressed on the surface of placental IE. We performed binding assays on many of the parasites used for the proteomics studies, and confirmed that placental parasites bind uniformly to CSA while parasites infecting children bind to diverse endothelial receptors other than CSA (Table 1).

P. falciparum adhesion occurs at points of contact between the endothelial surface and electron-dense protrusions called "knobs" that appear on the IE surface. Previous studies demonstrated that proteins associated with the knob structure are insoluble in non-ionic detergent but are soluble in ionic detergent. These properties allow sequential extraction of parasites with non-ionic then ionic detergents to enrich for knob-associated proteins including PfEMP1. We used this method to prepare IE membrane protein fractions from enriched parasite preparations, as previously described [13].

We performed shotgun proteomics on IE membrane fractions from 18 placental parasite samples and 21 parasite samples collected from the peripheral blood of children. In total, peptides corresponding to 2938 proteins were detected in placental parasites and 2161 proteins were detected in parasites from children. Twenty-six hypothetical proteins and 6 proteins with predicted functions were detected in two or more of the placental parasites but in none of the parasites from children.

Of the 26 hypothetical proteins detected only in placental parasites, 15 had been detected in earlier studies of laboratory isolates that do not bind CSA [11,12], suggesting that they are not unique to placental parasites. Of the remaining 11 hypothetical proteins, 10 had predicted TM domains. Four of these 11 proteins had strong evidence as distinct proteins of placental parasites (Table 2): three were found to be more abundant in placental parasites by AMT tag analysis (see below) and one protein (PF08_0046) was detected in four placental samples but no samples from children by MS/MS. Among the four hypothetical proteins with strong evidence for an association with placental parasites, one protein (PFI1785w) contains a *Plasmodium* export element (PEXEL/VTS) that targets proteins for translocation across the parasite vacuole membrane [24,25]. One of the proteins (PFD0690c) contains a putative signal sequence, suggesting that it may be transported, and potentially trafficked to the IE surface. None of these proteins was uniformly identified in placental parasite samples, and we speculate that this may be due to their relatively low abundance and the sensitivity limits of mass spectrometry. Conversely, we detected abundant membrane-associated proteins like KAHRP, PfEMP3 and glycophorin-binding protein 130 in all the samples that we tested.

Of the six proteins with predicted functions detected only in placental parasites by MS/MS, two are variant antigens. MS/MS

Table 1
Binding phenotype of studied parasites

Sample ID	Profile of binding receptors
Placenta parasites	
PI0034	CSA
PI0038b	CSA
PI0150	CSA
PI0222	CSA
PI0390	CSA
PI0405	CSA
PI0661	CSA
PI0687	CSA
PI0699	CSA
PI0711	CSA
PI0722	CSA
PI0747	CSA
PI0758	CSA
PI0863	CSA
PI0918	CSA
PI0961	CSA
PI0963	CSA
PI1000	CSA
PI01010	CSA
PI1020	CSA
Children parasites	
0063a01	CD36, ICAM-1, E-selectin, P-selectin
0083a01	CD36, TSP, ICAM-1, E-selectin, CD31, P-selectin
0093a01	CD36, CD31, P-selectin
0170a01	CD36, TSP, ICAM-1
0218a01	CD36, TSP, ICAM-1, E-selectin, CD31, P-selectin
0239a01	ICAM-1
0247a01	CD36, TSP, ICAM-1, E-selectin, CD31, P-selectin
0309a01	CD36, CD31, P-selectin
0321a01	CD36
0361a01	CD36, TSP, ICAM-1, E-selectin, CD31, P-selectin
0374a01	CD36
0397a01	CD36, TSP, ICAM-1, E-selectin, VCAM
0440a01	CD36, TSP
0446a01	CD31
0515a01	CD36, TSP, ICAM-1, CD31, P-selectin
0554a01	TSP, CD31
0597a01	CD36, TSP, ICAM-1, VCAM, CD31, P-selectin
0789a01	CD36, TSP
0480a01	ICAM-1, E-selectin, VCAM
0466a01	CD36
0220a01	CD36, E-selectin, VCAM, CD31, P-selectin
0413a01	CD36, TSP
0666a01	CD36, TSP
0199a01	CD36

detected two variants of VAR2CSA (see below) as well as a member of the rifin variant antigen family in membrane fractions from placental parasites but not other parasites. One protein with predicted function, the endoplasmin homolog (PFL1070c), was also found to be more abundant in placental parasites by AMT tag analysis. The other three proteins detected by MS/MS only in placental parasites were not confirmed in AMT tag analysis.

PFI1785w, a protein that appears to be abundant in the placental IE membrane fraction (Table 2), was recently suggested to be a variant antigen in comparative genomic hybridization (CGH) studies using microarrays [26]. Antigenic variation is an important immune evasion strategy of malaria parasites. However, PFI1785w is sometimes lost by deletion after continuous *in vitro* culture of laboratory isolates [27], which could also account for the CGH results. We determined PFI1785w sequences using cDNA from three placental parasite samples, and compared these to sequences in public databases. The sequence of PFI1785w was fully conserved among the placental parasites, suggesting that this antigen does not undergo significant variation. The sequence of PFI1785w in the 3D7 isolate contained two polymorphisms: a mutation at nucleotide position 133 (C → T) that converts methionine to threonine at residue 38, and a mutation at nucleotide position 605 (A → T) that leads to premature termination of the protein in 3D7 isolate. Because 3D7 parasites can only bind to CSA in a low numbers, we speculate that the truncated form of PFI1785w may interfere with the development of the CSA-binding phenotype. In microarray studies, PFI1785w transcripts have been detected in early trophozoite stage parasites of the HB3 isolate but not during blood stage development of 3D7 and Dd2 isolates (PlasmoDB expression data http://www.plasmodb.org). It is unknown whether PFI1785w may be expressed at the protein level in the HB3 isolate. In microarrays studies of the FCR3 isolate, PFI1785w was not detected in parasites selected to bind to either CD36 or CSA [28]. Similarly, our studies did not detect PFI1785w peptides in laboratory parasites selected to bind to CSA (data not shown). These results suggest that *in vivo* parasites express some IE surface proteins that are distinct from those of laboratory parasites, even when the different parasites display a similar binding phenotype.

3.2. *PfEMP1 detected by LC–MS/MS*

The best known IE surface protein is PfEMP1, a large, multidomain variant antigen encoded by a family of ~60 alleles (called *var* genes) in each haploid parasite genome. Despite

Table 2
Characteristics of proteins detected only in placental parasites by LC–MS/MS

Protein ID (PlasmoDB)	Placenta isolate ID#	No. of peptides	No. of spectra	Annotation	SP	TM	PEXEL/VTS
PF08_0046[a]	0038b; 0660; 0758; 1000	4	4	Hypothetical protein	n	y	n
PFB0870w[b]	0661; 0863; 0918	3	3	Hypothetical protein	n	y	n
PFD0690c[b]	0661; 0711	2	2	Hypothetical protein	y	y	n
PFI1785w[b]	0390; 0405; 0661; 0711; 0961; 0963; 1020	17	36	Hypothetical protein	n	y	y

[a] Samples analyzed by LC–MS/MS only.
[b] Data verified by MS-FTICR.

Table 3
VAR2CSA forms detected in different parasite types by LC–MS/MS

Reference	Parasite/source	Parasite type	No. of isolates	No. of peptides	Domain
gi|34525768; gi|34525760	IT var4; MC var6, [34]	Placenta	1	1	DBL5ε
gi|34525768; gi|34525760	IT var4; MC var6, [34]	Placenta	2	2	DBL4ε
gi|34525768; gi|34525760	IT var4; MC var6, [34]	Placenta	1	1	DBL1x
gi|34525768	IT var4, [34]	Placenta	1	1	DBL1x
gi|31323048	2O2, Salanti ([6])	Placenta	1	1	DBL2x
gi|31323048	2O2, Salanti ([6])	Non-pregnant	1	1	DBL2x
gi|31323049	2O2, Salanti ([6])	Non-pregnant	1	1	DBL3x
gi|31322999	MN39C1, Salanti ([6])	Non-pregnant	1	1	DBL3x
gi|31322987	MN47B1, Salanti ([6])	Non-pregnant	1	1	DBL2x

extensive *var* gene sequence variation within and between genomes, individual domains are recognizable that can be grouped according to relatively conserved motifs. The extracellular portion of each PfEMP1 protein contains between two to nine domains, and recombinant forms of individual domains support binding of different endothelial receptors *in vitro* [29].

var2csa (PFL0030c) encodes a relatively conserved PfEMP1 molecule that is significantly upregulated in placental parasites as well as laboratory isolates selected to bind CSA [6,30]. In our proteomics studies, peptides corresponding to VAR2CSA were detected in two placental parasite samples and in four samples from infected children (Table 3). All but one of the VAR2CSA peptides mapped to highly conserved regions of different DBL domains. The bias towards identifying conserved peptides probably reflects the requirement that exact sequence matches be available in the genome database. Peptides corresponding to VAR2CSA sequences from IT and MC isolates were detected in placental IE only, suggesting that these forms are preferentially expressed by placental parasites.

Previously, we reported that peptides corresponding to VAR2CSA were present in IE membrane fractions of placental parasite isolates from Kenya that had been adapted to *in vitro* culture [13]. In those studies, VAR2CSA was detected before and after the parasites had lost their ability to bind CSA. Another study reported that *var2csa* is transcribed at relatively high levels in some parasite samples collected from children [31] as well as a laboratory isolate that had "rosetting" properties (i.e., bound uninfected erythrocytes) [32]. Taken together, the different studies suggest that *var2CSA* can be detected at the level of both transcript and protein in parasites with varying binding properties and from various parasite donors, including parasites collected from children that do not bind to CSA.

3.3. Parasite proteins expressed at higher levels in placental IE by FTICR

We quantified protein abundance using data from FTICR MS, and examined differences in the expression of IE surface proteins between placental parasites and parasites from children. Peptides identified by LC–MS/MS were used to create a PMT (potential mass and time tag) library. LC-FTICR MS analysis of the peptides in the PMT library provided an accurate mass tag and time (AMT) dataset. The FTICR MS results are highly accurate, which allows quantification of protein abundance [14,21]. The average peak intensities of peptides that correspond to a protein are used to calculate its abundance. FTICR detection also provides a method for validating the LC–MS/MS peptide identifications.

The abundance of each protein in the proteome was estimated, establishing the quantitative proteome for each sample. We examined the reproducibility of this approach by repeating the assay two to four times on each parasite sample. Assay results were normalized and clustered according to the abundance of detected proteins, and in all cases replicate assays on the same sample clustered together most closely (Fig. 1). The average correlation between replicate assays of the same samples was >0.9 (Spearman rank correlation test), reflecting the high reproducibility of this approach.

Overall, FTICR MS detected a greater number of proteins exclusively or preferentially expressed by placental parasites, compared to LC–MS/MS, and this is consistent with the greater sensitivity of FTICR MS. Protein abundance was calculated from the average abundance values of the corresponding peptides in each FTICR MS analysis [16]. These values were normalized using a cluster algorithm [22]. In statistical analyses, 50 proteins were more abundant in placental parasites than in parasites from children (Table 4 and Supplementary Table 1). Of these, 20 proteins were detected in three or more placental samples, including 3 PfEMP1 proteins and 17 hypothetical proteins (Table 4), and all of these have putative TM domains. The 17 hypothetical proteins included 6 that were exclusively expressed, and 11 that were preferentially expressed, by placental parasites.

In earlier yeast two-hybrid screens [33], several of the proteins that we detected at higher levels in placental parasites were found to interact with each other (Table 5). Interactions between upregulated proteins may indicate that protein networks are involved in the production of the placental parasite phenotype, and that upregulation of a single protein like VAR2CSA to mediate adhesion to CSA may not fully recapitulate the placental parasite phenotype.

PfEMP1 peptides that were more abundant in placental parasites by FTICR analysis correspond to three PfEMP1 proteins (Table 4), including a form of VAR2CSA from the MC isolate of *P. falciparum*. In earlier studies, these three PfEMP1 molecules were also detected in sporozoites [12], as well as blood stage parasites with and without the CSA-binding phenotype [13]. Among these PfEMP1 molecules, we find that PFE0005w was

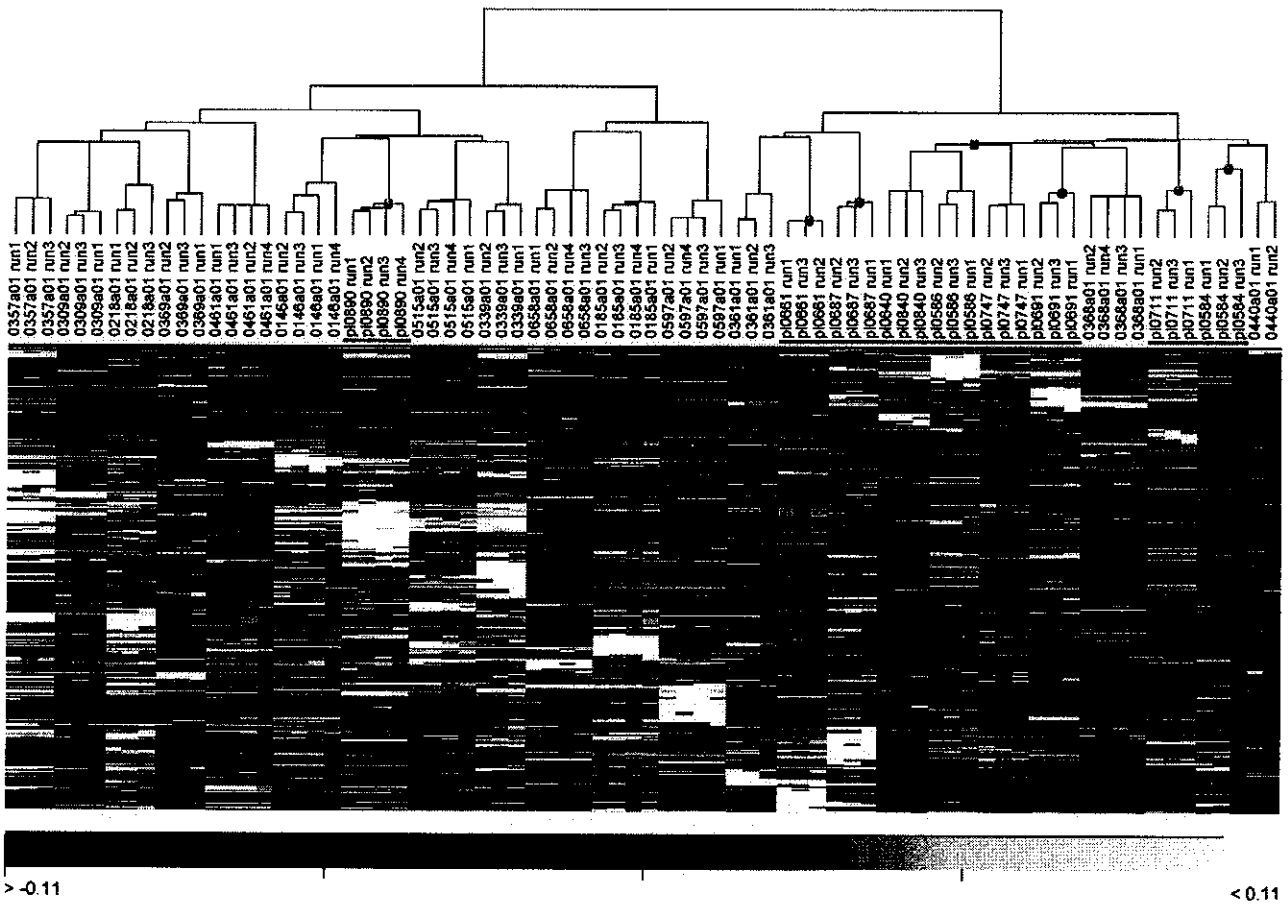

Fig. 1. Hierarchical clustering of 820 *Plasmodium falciparum* proteins detected by AMT tag analysis was performed using Acuity 4.0 (Axon). Data were normalized using Cluster program (http://rana.lbl.gov/EisenSoftware.htm). Placental parasite samples are underlined.

Table 4
Proteins associated with placental parasites by quantitative proteomics

Protein ID	Annotation	*p*-Value (Mann–Whitney *U* test)
PF13_0003[a]	PfEMP1	0.003
PFE0005w[b]	PfEMP1	0.0009
MC var 6[a]	PfEMP1 (VAR2CSA)	0.003
PF10_0232[a]	Hypothetical protein	≤.0001
PF11_0437[a]	Hypothetical protein	0.004
PF13_0162[a]	Hypothetical protein	0.01
PF14_0016[a]	Hypothetical protein	0.004
PF14_0260[a]	Hypothetical protein	0.003
PF14_0507[a]	Hypothetical protein	≤.0001
PF14_0616[b]	Hypothetical protein	0.001
PFA0700c[b]	Hypothetical protein	0.007
PFB0115w[a]	Hypothetical protein	≤.0001
PFB0870w[b]	Hypothetical protein	0.02
PFB0888w[a]	Hypothetical protein	0.01
PFC0245c[a]	Hypothetical protein	0.0002
PFC0715c[b]	Hypothetical protein	≤.0001
PFC0850c[b]	Hypothetical protein	≤.0001
PFD0690c[b]	Hypothetical protein	≤.0001
PFI1785w[a]	Hypothetical protein	0.01
PFL2505c[a]	Hypothetical protein	0.004

[a] Protein more abundant in placental parasites.
[b] Protein exclusively expressed by placental parasites.

expressed only in placental parasites, while PF13_0003 and VAR2CSA were expressed in both placental parasites and children's parasites. Two forms of VAR2CSA (matching sequences found in the genomes of the Dd2 isolate and the IT isolate, respectively) were expressed at similar levels by placental parasites versus children's parasites.

Several placental parasite samples expressed more than one form of PfEMP1. This may not seem surprising, since many *in vivo* infections are polyclonal. However, the finding does not seem consistent with the idea that a single relatively conserved PfEMP1 mediates binding of placental parasites, which in these studies uniformly adhere to the receptor CSA (Table 1). Furthermore, the diversity of PfEMP1 forms in these samples may be

Table 5
Interactions involving proteins associated with placental IE

Protein ID	Annotation	Interacting proteins ID
PF10_0232	Hypothetical protein	MAL7P1.155
		PF07_0106
		PF11_0191
PFB0115w	Hypothetical protein	MAL13P1.336
PFC0245c	Hypothetical protein	PFD1175w
		PFL0190w
PFL2505c	Hypothetical protein	PFB0640c

greater than we have described here. MS studies of PfEMP1 are limited by the lack of representation of all possible variant forms in the database. Even relatively conserved regions of PfEMP1 display sufficient variation to reduce the likelihood of peptide identification, since MS requires exact sequence matches. This will limit our ability to identify the full repertoire of PfEMP1 expressed on the IE surface.

In addition to the parasite molecules described above, an additional 30 proteins (Supplementary Table 1) were expressed exclusively or preferentially by placental parasites, but were detected in only one or two samples. Of these 30 proteins, 22 contained motifs that might suggest trafficking or membrane localization (signal peptide, TM domain, PEXEL sequence). Another two proteins that were upregulated in placental parasites had known functions that did not suggest a role as a surface antigen. However, we do not exclude that some of these proteins may be IE surface proteins, or may play a role in the development of the placental parasite phenotype.

3.4. PFI1785w expression in placental IE

In proteomics studies, PFI1785w was commonly detected in placental IE membrane fractions. To confirm that PFI1785w is specifically expressed in placental parasites, we raised antibodies in mice against recombinant protein expressed in *E. coli*. Anti-PFI1785w antisera reacted with a protein of the predicted size of 42 kDa in membrane fractions of placental IE but not IE from children (Fig. 2). Pre-immune sera did not react with IE membrane fractions (not shown). Knob-associated histidine rich protein (KAHRP) was recognized in extracts of both placental parasites and parasites from children.

In immunolocalization assays, antisera against PFI1785w failed to react with live or paraformaldehyde-fixed placental IE, suggesting that the antibodies raised against recombinant PFI1785w are directed to denatured protein but not to conformational epitopes. Monoclonal antibody to KAHRP reacted with placental IE membranes in fixed but not live samples, consistent with its association with the internal face of the IE membrane.

3.5. Real time PCR

None of the proteins identified in this study were uniformly detected in all placental parasites, although placental IEs uniformly demonstrated binding to CSA. Despite substantial improvements in recent years, proteomics tools have limited sensitivity. As a complementary approach to validate the MS findings, we used qPCR to measure the abundance of transcripts corresponding to the proteins that we had identified by proteomics tools. We compared levels of transcription between six placental parasites and six children's parasites cultured *in vitro* to develop into mature parasite forms. PFI1785w and PFB0115w were transcribed at 10- and 7-fold higher levels, respectively in placental parasites. Transcript levels of PFD0690c were similar in parasite samples from placentas versus those from children. The discordance between transcript and protein levels of PFD0690c may indicate that the regulation of this protein does not occur at the level of transcription. Alternatively, the discordance may reflect a technical issue, for example that differential transcription of this gene may need to be measured at a different timepoint in the life cycle. In a recent comparative transcriptome study (Francis et al., submitted for publication), PFI1785w and PFB0115w were highly upregulated in placental parasites compared to parasites from children, in agreement with our proteomic studies. In a comparative transcriptome study of two isogenic 3D7 *P. falciparum* laboratory clones with distinct adhesive properties, PFB0115w was transcribed at higher levels in the clone that rosetted at high levels, but this difference was mainly seen during ring stage development stage [32]. In the present study, PFB0115w were detected in some IE from children, but the expression of this protein was significantly higher in placental IE by AMT tag analysis.

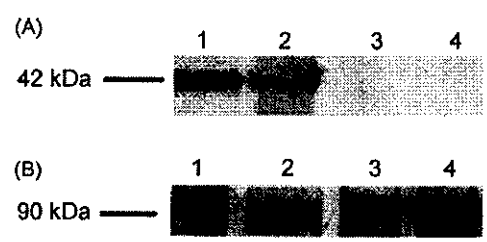

Fig. 2. Western blot analysis was performed using mouse anti-PFI1785w antisera (A) and anti-KAHRP monoclonal antibody (B). Lanes 1 and 2, membrane extracts from placental parasites; lanes 3 and 4, membrane extracts from children's parasites. MW markers are indicated on the left.

4. Conclusions

In this study, we used conventional MS/MS methodology as well as novel AMT tag technology to identify proteins that are exclusively or preferentially expressed by placental parasites. The study focused on IE membrane proteins, which are likely to be involved in host–parasite interactions. In this combined approach, we identified seven hypothetical proteins that are exclusively detected in placental parasites, and were found in three or more placental samples. This is the first study to confirm the expression of these genes at the protein level. All the proteins contain a putative TM domain, four contain a predicted signal sequence, and two contain PEXEL/VTS sequences suggesting translocation across the parasite vacuole membrane. The conserved protein PFI1785w appeared to be particularly abundant in placental parasites, at both the transcript and protein level. Further studies should examine the effect of PFI1785w disruptions on the ability of parasites to develop the CSA-binding phenotype and the localization of the protein by immunofluorescence studies on live parasites and immuno-electron microscopy studies using antibody that recognizes native protein.

VAR2CSA is currently a leading candidate for a pregnancy malaria vaccine. Earlier studies reported that transcription of the *var2csa* gene is upregulated in CSA-selected as well as placental parasites, and that antibody levels against VAR2CSA increase over successive pregnancies [30]. However, surface

expression of this protein in placental parasites has not yet been confirmed. We identified peptides corresponding to VAR2CSA in membrane fractions of non-CSA-binding parasites from children as well as CSA-binding placental parasites. Specific VAR2CSA forms appeared to be upregulated in placental parasites, while other forms appeared to be expressed at similar levels in placental versus children's samples. Future studies should examine the VAR2CSA variants and other membrane-associated proteins identified in these studies for their separate roles in placental malaria pathogenesis and protective immunity.

Acknowledgements

This work was supported by grants from the US National Institutes of Health (grant AI52059 to P.E.D.) and the Bill & Melinda Gates Foundation (grant 3035 to P.E.D.). The authors wish to thank MOMS Project nurses and laboratory staff stationed at Muheza DDH who collected and processed the samples used in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.05.010.

References

[1] David PH, Hommel M, Miller LH, Udeinya IJ, Oligino LD. Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. Proc Natl Acad Sci USA 1983;80:5075–9.

[2] Fried M, Muga RO, Misore AO, Duffy PE. Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. J Immunol 1998;160:2523–30.

[3] Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science 1996;272:1502–4.

[4] Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature 1998;395:851–2.

[5] Duffy PE, Fried M. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. Infect Immun 2003;71:6620–3.

[6] Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol Microbiol 2003;49:179–91.

[7] Tuikue Ndam NG, Salanti A, Bertin G, Dahlback M, Fievet N, Turner L, et al. High level of var2csa transcription by *Plasmodium falciparum* isolated from the placenta. J Infect Dis 2005;192:331–5.

[8] Gamain B, Trimnell AR, Scheidig C, Scherf A, Miller LH, Smith JD. Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. J Infect Dis 2005;191:1010–3.

[9] Sam-Yellowe TY, Florens L, Johnson JR, Wang T, Drazba JA, Le Roch KG, et al. A Plasmodium gene family encoding Maurer's cleft membrane proteins: structural properties and expression profiling. Genome Res 2004;14:1052–9.

[10] Florens L, Liu X, Wang Y, Yang S, Schwartz O, Peglar M, et al. Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. Mol Biochem Parasitol 2004;135:1–11.

[11] Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, Sauerwein RW, et al. Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry. Nature 2002;419:537–42.

[12] Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. Nature 2002;419:520–6.

[13] Fried M, Wendler JP, Mutabingwa TK, Duffy PE. Mass spectrometric analysis of *Plasmodium falciparum* erythrocyte membrane protein-1 variants expressed by placental malaria parasites. Proteomics 2004;4:1086–93.

[14] Smith RD, Anderson GA, Lipton MS, Pasa-Tolic L, Shen Y, Conrads TP, et al. An accurate mass tag strategy for quantitative and high-throughput proteome measurements. Proteomics 2002;2:513–23.

[15] Adkins JN, Monroe ME, Auberry KJ, Shen Y, Jacobs JM, Camp DG, 2nd, et al. A proteomic study of the HUPO Plasma Proteome Project's pilot samples using an accurate mass and time tag strategy. Proteomics 2005; 5: 3454-66.

[16] Hixson KK, Adkins JN, Baker SE, Moore RJ, Chromy BA, Smith RD, et al. Biomarker candidate identification in yersinia pestis using organism-wide semiquantitative proteomics. J Proteome Res 2006;5:3008–17.

[17] Ding YH, Hixson KK, Giometti CS, Stanley A, Esteve-Nunez A, Khare T, et al. The proteome of dissimilatory metal-reducing microorganism *Geobacter sulfurreducens* under various growth conditions. Biochim Biophys Acta 2006;1764:1198–206.

[18] Mutabingwa TK, Bolla MC, Li JL, Domingo GJ, Li X, Fried M, et al. Maternal malaria and gravidity interact to modify infant susceptibility to malaria. PLoS Med 2005;2:1260–8.

[19] Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673–5.

[20] Qian WJ, Liu T, Monroe ME, Strittmatter EF, Jacobs JM, Kangas LJ, et al. Probability-based evaluation of peptide and protein identifications from tandem mass spectrometry and SEQUEST analysis: the human proteome. J Proteome Res 2005;4:53–62.

[21] Lipton MS, Pasa-Tolic L, Anderson GA, Anderson DJ, Auberry DL, Battista JR, et al. Global analysis of the *Deinococcus radiodurans* proteome by using accurate mass tags. Proc Natl Acad Sci USA 2002;99:11049–54.

[22] Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998;95:14863–8.

[23] Rock EP, Saul AJ, Taylor DW, Leech JH, Sherwood J, AHoward RJ. Expression of the histidine-rich protein PfHRP1 by knob-positive *Plasmodium falciparum* is not sufficient for cytoadherence of infected erythrocytes. Infect Immun 1988;56:3301–4.

[24] Marti M, Good RT, Rug M, Knuepfer E, Cowman AF. Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science 2004;306:1930–3.

[25] Hiller NL, Bhattacharjee S, van Ooij C, Liolios K, Harrison T, Lopez-Estrano C, et al. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science 2004;306:1934–7.

[26] Kidgell C, Volkman SK, Daily J, Borevitz JO, Plouffe D, Zhou Y, et al. A systematic map of genetic variation in *Plasmodium falciparum*. PLoS Pathog 2006;2:pe57.

[27] Shirley MW, Biggs BA, Forsyth KP, Brown HJ, Thompson JK, Brown GV, et al. Chromosome 9 from independent clones and isolates of *Plasmodium falciparum* undergoes subtelomeric deletions with similar breakpoints in vitro. Mol Biochem Parasitol 1990;40:137–45.

[28] Ralph SA, Bischoff E, Mattei D, Sismeiro O, Dillies MA, Guigon G, et al. Transcriptome analysis of antigenic variation in *Plasmodium falciparum*—var silencing is not dependent on antisense RNA. Genome Biol 2005;6:R93.

[29] Kraemer SM, Smith JD. A family affair: var genes, PfEMP1 binding, and malaria disease. Curr Opin Microbiol 2006;9:374–80.

[30] Salanti A, Dahlback M, Turner L, Nielsen MA, Barfod L, Magistrado P, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med 2004;200:1197–203.

[31] Duffy MF, Caragounis A, Noviyanti R, Kyriacou HM, Choong EK, Boysen K, et al. Transcribed var genes associated with placental malaria in Malawian women. Infect Immun 2006;74:4875–83.

[32] Mok BW, Ribacke U, Winter G, Yip BH, Tan CS, Fernandez V, et al. Comparative transcriptomal analysis of isogenic *Plasmodium falciparum* clones of distinct antigenic and adhesive phenotypes. Mol Biochem Parasitol 2007;151:184–92.

[33] LaCount DJ, Vignali M, Chettier R, Phansalkar A, Bell R, Hesselberth JR, et al. A protein interaction network of the malaria parasite *Plasmodium falciparum*. Nature 2005;438:103–7.

[34] Kraemer SM, Smith JD. Evidence for the importance of genetic structuring to the structural and functional specialization of the *Plasmodium falciparum* var gene family. Mol Microbiol 2003;50:1527–38.

INFECTION AND IMMUNITY, Oct. 2007, p. 4838–4850 Vol. 75, No. 10
0019-9567/07/$08.00+0 doi:10.1128/IAI.00635-07

Six Genes Are Preferentially Transcribed by the Circulating and Sequestered Forms of *Plasmodium falciparum* Parasites That Infect Pregnant Women[▽]†

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Received 4 May 2007/Returned for modification 17 June 2007/Accepted 1 August 2007

In areas of stable malaria transmission, susceptibility to *Plasmodium falciparum* malaria increases during first pregnancy. Women become resistant to pregnancy malaria over successive pregnancies as they acquire antibodies against the parasite forms that sequester in the placenta, suggesting that a vaccine is feasible. Placental parasites are antigenically distinct and bind receptors, like chondroitin sulfate A (CSA), that are not commonly bound by other parasites. We used whole-genome-expression analysis to find transcripts that distinguish parasites of pregnant women from other parasites and employed a novel approach to define and adjust for cell cycle timing of parasites. Transcription of six genes was substantially higher in both placental parasites and peripheral parasites from pregnant women, and each gene encodes a protein with a putative export sequence and/or transmembrane domain. This cohort of genes includes *var2csa*, a member of the variant PfEMP1 gene family previously implicated in pregnancy malaria, as well as five conserved genes of unknown functions. Women in East Africa acquire antibodies over successive pregnancies against a protein encoded by one of these genes, *PFD1140w*, and this protein shows seroreactivity similar to that of VAR2CSA domains. These findings suggest that a suite of genes may be important for the genesis of the placental binding phenotype of *P. falciparum* and may provide novel targets for therapeutic intervention.

By adulthood, individuals living in areas of stable malaria transmission acquire clinical immunity as a result of repeated infection with *Plasmodium falciparum* (11, 41, 42). However, women pregnant for the first time become susceptible to infection by antigenically distinct parasites that sequester in the placenta (6, 17), producing a specific malaria syndrome known as placental malaria, pregnancy malaria, or pregnancy-associated malaria. Pregnancy malaria commonly causes severe maternal anemia, low birth weight, and increased neonatal and infant mortality. The deaths of between 100,000 and 200,000 African infants each year are attributed to malaria during pregnancy, making this an enormous public health burden (27).

Placental parasites have distinct properties that suggest a unique repertoire of surface antigens. Infected erythrocytes (IE) in the placenta adhere to low-sulfated forms of chondroitin sulfate A (CSA), a glycosaminoglycan found on the surface of the syncytiotrophoblast (1, 2, 20, 22, 25) and throughout the intervillous spaces of infected placentas (47). Although the distribution of CSA on vascular surfaces has not been fully defined, CSA binding appears to be largely associated with parasites from pregnant women (18, 57). Conversely, placental parasites do not typically bind CD36, ICAM-1, or other endothelial receptors (17, 57) that commonly support binding of parasites associated with other malaria syndromes (5, 6, 28, 64).

The frequency and severity of placental malaria decrease over successive pregnancies, as women acquire adhesion-blocking antibodies against placental IE (21). Serum immunoglobulin G (IgG) from multigravid women living in areas of endemicity has been shown to block adhesion of placental or CSA-selected parasites collected from different continents (18, 21, 54, 66). Adhesion-blocking antibodies are not detected in males or women before first pregnancy. This pattern of naturally acquired immunity is consistent with repeated exposure to a finite number of antigens during pregnancy that are not seen in childhood infections, raising expectations that a vaccine can be developed once the IE surface antigens of placental parasites are identified.

The search for IE surface antigens of placental parasites has focused on the *var* gene family. *var* genes encode antigenically varied 200- to 400-kDa multidomain IE surface proteins called PfEMP1 that have been implicated in adhesion to other receptors (62, 65). There are 59 *var* genes in the 3D7 *P. falciparum* genome (24) and probably similar numbers in other strains (33).

var gene variants *varCS2* (53) and *FCR3.varCSA* (*varIcsa*) (10) have both been linked to CSA binding in studies of parasites selected in vitro. Deletion of *FCR3.varCSA* resulted in

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▽ Published ahead of print on 13 August 2007.

initial loss of CSA binding, suggesting a role for VAR1CSA in binding of the FCR3 parasite line to CSA. However, other studies suggest that *var1csa* is not central to adhesion of placental parasites. Transcription of *var1csa* is not restricted to placental parasites (19), and seroreactivity to expressed VAR1CSA is not parity related (31, 50). After selection on CSA, the FCR3 knockout parasite regained binding to CSA and expressed a novel PfEMP1 gene, subsequently shown to be *var2csa* (3, 23).

A comprehensive analysis of *var* gene transcription in CSA-selected NF54 laboratory parasites revealed that *PFL0030c*, now known as *var2csa*, was transcribed at high levels (60). Transcription of *var2csa* has been confirmed in other CSA-selected laboratory strains as well as isolates from pregnant women (13, 15). *var2csa* was recently shown to be the most highly expressed *var* gene detected in placental parasites from Malawi, although one placental parasite sample preferentially expressed the DBL2γ domain of *varCS2* (14). CSA-selected parasites expressing *var2csa* are recognized by sera from Ghana and Senegal in a parity- and gender-specific manner (60, 67) that correlates with protection.

The mass of data accumulated over the last few years is consistent with the hypothesis that VAR2CSA is the dominant PfEMP1 associated with parasite adhesion to the placenta. However, fundamental gaps remain in our understanding of placental parasites. While VAR2CSA has been shown to be localized to the IE cell surfaces of CSA-selected parasites (4, 15), there are no published reports of VAR2CSA cell surface localization in parasites from pregnant women. Cross-linking experiments examining binding of IE surface proteins to CSA showed that an unidentified 22-kDa protein and not VAR2CSA bound to CSA (26), and no studies have demonstrated that soluble VAR2CSA can inhibit binding of placental parasites. These findings raise the possibility that other proteins in addition to VAR2CSA are required for the placental parasite phenotype, for example, as part of a multimeric protein complex at the IE surface (26), which could also reconcile the biochemical properties of PfEMP1 with its membrane topology (51). PfEMP1 is synthesized as a peripheral membrane protein (51) and appears to be part of a complex, rather than in vesicles in the IE cytosol (32), prior to localization to the Maurer's cleft and subsequent insertion in the erythrocyte membrane (35, 43, 51).

Since the erythrocyte lacks a protein transport apparatus, many of the necessary components must be exported by the parasite. The predicted secretome responsible for erythrocyte remodeling, protein transport, and adhesion properties is composed of about 400 proteins with sorting motifs that direct proteins beyond the parasitophorous vacuole membrane plus additional proteins that lack these motifs (7, 12, 29, 30, 44, 48, 63). Many secretome proteins are encoded by members of paralogous families with unknown functions that show dramatic expansion in *P. falciparum* and may define specific virulence properties.

In an effort to characterize the placenta binding phenotype of clinical parasites and to identify novel antigens that are expressed by parasites causing infections, we examined the whole-genome transcriptional profiles of parasites collected from Tanzanian women with pregnancy malaria. Parasites were profiled either directly after collection in the clinic or after a maximum of 20 h in culture so that gene expression patterns characteristic of parasites causing disease would be maintained. Both circulating early-stage parasites and sequestered late-stage parasites from women with pregnancy malaria were analyzed so that the transcriptional profiles of parasites just prior to and during sequestration could be determined. We found that increased *var2csa* expression is always observed in maternal parasites at both stages of intraerythrocytic development and that a suite of five additional genes, all with unknown functions, is also consistently up-regulated by these parasites.

MATERIALS AND METHODS

Clinical sample collection and parasite adhesion assays. All samples were collected from study participants in the ongoing Mother Offspring Malaria Studies (MOMS) Project carried out in Muheza district, northeastern Tanzania, an area of intense malaria transmission as previously described (16, 46). Women provided signed, informed consent for themselves and their children to participate in the study and to provide samples for parasite studies. Study procedures involving human participants were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the U.S. National Institutes of Health, and ethical clearance was obtained from the Institutional Review Board of Seattle Biomedical Research Institute and the National Medical Research Coordinating Committee in Tanzania.

Placental parasites were obtained as previously described (17). Parasites from the peripheral circulation of mothers and children were cultured and tested for adhesion to immobilized purified receptors, including CSA, hyaluronic acid (HA), thrombospondin (TSP), ICAM-1, CD36, and bovine serum albumin (BSA) (control). Assays were performed within 24 h of sample collection (16).

RNA preparation and microarray hybridization. Intervillous blood was obtained by mechanical grinding of placentas, and intraerythrocytic parasites were released from red blood cells by saponin lysis. The released parasites were stored in RNA Later solution (Ambion) at −20°C and subsequently solubilized in TRIzol (Invitrogen). Peripheral parasites from mothers and children were collected and stored in either TRIzol or RNA Wiz (Ambion) and stored at −80°C. Peripheral parasites from children were cultured for 20 h until parasites matured to the trophozoite stage, solubilized in TRIzol, and stored at −80°C.

RNA was isolated according to the manufacturer's instructions. Microarray probes were prepared using a MessageAmpII aminoallyl labeling kit, starting with 1 to 3 μg of total RNA from each sample, following the kit instructions (Ambion). Probes were coupled to Cy3 and Cy5 monoesters (GE Healthcare Biosciences). Probe samples to be compared were combined and fragmented with RNA fragmentation reagent (Ambion).

All slides were prehybridized at 63°C with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml BSA for 60 min to 4 h and then washed in nuclease-free water and dried. The hybridization buffer was 3× SSC, 25 mM HEPES (pH 7.5), 0.75 mg/ml poly(A) DNA (Sigma), 0.2% SDS. Differential gene expression was determined by direct comparison of 5 μg of a labeled probe from maternal-parasite RNA to 5 μg of a labeled probe with comparable specific activity from child-derived-parasite RNA. Multiple comparisons were made for each clinical parasite sample. Slides were hybridized at 63°C for 16 to 20 h and washed in 2× SSC, 0.2% SDS, followed by a wash in 0.1× SSC.

Data were acquired with GenePix Pro 4000A (Molecular Devices) and analyzed using GenePix 5.1 (Axon Instruments) and Acuity 4.0 (Molecular Devices) software. Data were normalized using a locally weighted linear regression algorithm without background subtraction. Unflagged spots whose background-subtracted intensities were 3 standard deviations above the local background level were analyzed.

Microarray fabrication. Microarrays were prepared by spotting the *P. falciparum* genome set of 70-mer oligonucleotides (Operon Biotechnologies) and 396 PfEMP1 70-mer oligonucleotides. These include the primary 3D7 elements from the Operon set, additional domain-specific elements designed for selected 3D7 *var* genes, elements from sequences obtained from field studies, the conserved regions from type A PfEMP1 genes implicated in severe malaria (37), and consensus *var2csa* elements designed by aligning 3D7, IT4, and DD2 strains of *P. falciparum*. In total, 4,872 3D7 *P. falciparum* genes were represented by oligonucleotide probes. Custom oligonucleotides were designed manually or using OligoArraySelector software and then synthesized (Illumina). All oligonucleotides were resuspended in 3× SSC to a concentration of 50 μM and spotted in duplicate on each UltraGapII slide (Corning), using a Gene Machine GR-04

TABLE 1. Real-time-PCR primers

Gene	Forward primer	Reverse primer
PFB0115w	5′-AAGGTATACCAAAGGAACAACC-3′	5′-CATCAACATGTGGTTCTTGTC-3′
PFB0115w	5′-AGAACAACAGAATTATCAACTACATC-3′	5′-TGTTCCTTTGGTATACCTTGTG-3′
PFD1140w	5′ATGAAGCGTTGAATCTTTTATC-3′	5′-TTTTCACTCTTACATAATTCATTCC-3′
PFD1140w	5′-GATATTGATGATATTGAAGAAGAGG-3′	5′-AAATCATTATAAGTTCCGGATG-3′
PFI1785w	5′-ATCTGCAGGTAGATATTCATGG-3′	5′-AAATTCTTAAAGCGGTTTTACC-3′
PFI1785w	5′-GAAATGAAGAAAGGAAATTTAGC-3′	5′-TTTTAAGGGTTTCTTCACATTC-3′
PFF0435	5′-CAAGGAGAAGCTGGTGTTATAG-3′	5′-AACCTGTTTGTACTTCATCTGC-3′
PFF0435	5′-TGATAAATTAGGTGCTCCATTC-3′	5′-AGATATCCCATACGTTGACAAG-3′
PFL0050c	5′-AAATTCAGTAAGAATTGCATGG-3′	5′-TCATCTACGTCTTCAATTTCATC-3′
PFL0050c	5′-ATGAATGTCGTCATATTGTTCC	5′-AGCTTTCCATGCAATTCTTAC-3′
MAL7P1.225	5′-TAGCTAAAGATGGATTTGATGG-3′	5′-TTGATTTATACCATGTTTCACG-3′
MAL7P1.225	5′-ATGGATTTGATGGTATGTTGAC-3′	5′-TGATGGTATGTTGACAGAATTATC-3′
PF10–0013	5′-TTTAGGAGATTCATTGAAGAGAAC-3′	5′-TCAGCATATCTTTCGTTCATAAG-3′
PF10–0013	5′-ATATCGGAACAAACTTCTGATG-3′	5′-GATTGTAATTCATGATTGTCCTC-3′
PF07_0073	5′- AAGTAGCAGGTCATCGTGGTT-3′	5′-TTCGGCACATTCTTCCATAA-3′
PF14_0425	5′-TGTACCACCAGCCTTACCAG-3′	5′-TTCCTTGCCATGTGTTCAAT-3′

Omnigrid oligonucleotide arrayer (Gene Machines). Slides were cross-linked and blocked using protocols from http://derisilab.ucsf.edu.

Quantitative reverse transcription-PCR (Q-PCR). DNA was removed from RNA with Turbo DNase (Ambion), following the kit instructions. RNA was reverse transcribed for 2 h at 46°C using Superscript III (Invitrogen) and primed with random nonamers (Sigma Aldrich). RNA from ring stage parasites was prepared by amplification using a Message Amp II kit (Ambion). PCR primers for selected genes were designed using Primer3 software (58). Real-time PCR was performed with the 7500 fast real-time PCR system (Applied Biosystems) for 45 cycles at 59°C using a SYBR green master mix kit (Applied Biosystems) and 0.5 mM primers. PCRs were performed in triplicate. Relative quantitation of RNA expression was done using the $\Delta\Delta CT$ method (user bulletin 2; Applied Biosystems), using seryl-tRNA synthetase and fructose biphosphate aldolase as constitutive controls (60). Maternal samples were compared to samples from children, which served as the calibrator. *P* values were determined using the Kruskal-Wallis algorithm. Primer pairs used in this study are listed in Table 1.

Cell cycle mapping. Hourly microarray studies encompassing the 48-h HB3 blood stage cycle (8) were used to map the developmental timing of clinical samples. We determined that 1,847 elements had valid spots across 51 of 55 microarrays in the HB3 series (i.e., all arrays other than TP_1a, TP_7b, TP_11a, and TP_39). The intensity of each element at each time point was divided by total intensity of Cy5 for the corresponding time point, and the logarithm was taken from the result of the division. Clinical sample microarrays were treated similarly. Elements common to both datasets were used to calculate both Euclidean and correlation distances between datasets. Correlation distances were defined as 1 minus the correlation coefficient. Euclidean distances were centered by deducting the mean of all 51 distances and plotted.

Multiple regression analysis. For each probe, the $\log_2$ intensities of all samples were calculated from microarray ratios by using multiple regression analysis. The design matrix was designated with values 1 and −1 in positions where samples were arrays in Cy5 and Cy3, respectively. *P* values corrected for multiple testing were calculated according to the Westfall-Young algorithm (69).

Hierarchical clustering of timing profiles. Timing profiles in correlation distances from different microarray slides from the same clinical samples were calculated, and their means were used for cluster analysis. Hierarchical clustering was performed using Euclidean distances and an average linkage algorithm.

PCA of the blood stage cycle. The 51 HB3 blood stage microarrays with valid spots (see above), plus all clinical microarrays from the current study, were normalized by total intensity and log transformed. If valid signals for a particular element existed in at least half of the arrays for a clinical sample, their mean value was used as the intensity of the element. Between 51 blood stage arrays and 20 clinical samples, there was a data set of 1,554 shared elements. Principal-component analysis (PCA) of the 51 blood stage time points (restricted to the described 1,554-element data set) showed that the first three components can be retained, and they explain 93% of the variance. After varimax rotation, these three principal components corresponded to the late ring-early trophozoite, late trophozoite-early schizont, and late schizont-early ring stages (see Fig. S3 in the supplemental material). Loading of clinical samples on the principal components was calculated as a correlation coefficient between each clinical sample and the principal components.

PfEMP1 gene expression analysis. All microarray elements were blasted against 97 PfEMP1 genes, pseudogenes, and truncated genes from the 3D7 strain. Partial homology with an *E* score of 6e−10 or less was considered a hit (see Table S3 in the supplemental material). Three hundred ninety-six elements were selected. The intensity of each element in each array was divided by the total intensity for the corresponding array. If the intensities of duplicate spots differed twofold or more, both spots were considered invalid. Otherwise, their average value was taken. If any array had an element whose intensity differed more than threefold from the mean intensity of the element for that clinical sample across all applicable arrays, then that element was excluded from further analysis. After this step, 377 elements had consistent measurements across all clinical samples. The switched-off expression level of a particular element was calculated as the mean expression level of the three lowest-expressing clinical samples (out of 11) of the same element. As a compromise between sensitivity in detection of switched-on expression and reduction of false scoring due to low-homology cross-hybridization by other PfEMP1 genes, we counted an element as switched on in a clinical sample only if its mean expression level exceeded its switched-off expression level eightfold or more.

Cloning and expression of PFD1140w. cDNA encoding the predicted extracellular portion of PFD1140w (amino acid residues 34 through 347) was amplified using genomic 3D7 DNA and PCR with two primers: forward (CCCCTCG AGATGGACTACAAAGACGATGACGATAAGAAAAGTTCTAGTGGCT CATGTGTTAATAG) and reverse (CCCGGATCCTTAATTAGACAAAGCA TCAATAATTTTTTTTTC). The forward primer introduced the coding sequence for the FLAG epitope. The PCR fragment was cloned into the XhoI and BamHI restriction sites of the pEU-E01-MCS vector (CellFree Sciences). Transcription and translation in the wheat germ cell-free system were performed according to the manufacturer's instructions. To confirm synthesis, synthetic protein was purified on anti-FLAG resin (Sigma) by using 3× FLAG peptide and analyzed by SDS gel electrophoresis and Coomassie blue staining (see Fig. 5A).

Seroepidemiology of PFD1140w. Mock and PFD1140w translation mixtures were diluted 1:10 with Superblock (Pierce) and incubated (100 μl per well) in anti-FLAG 96-well plates (Sigma) overnight at 4°C for rapid immobilization/purification of antigen. Human sera (from regions of malaria endemicity as well as from nonimmune individuals from the United States) were preincubated overnight at 4°C with equal volumes of 10 mg/ml normal mouse IgG and then diluted 1:100 and added to enzyme-linked-immunosorbent-assay plates. After 2 h of incubation at room temperature, wells were washed with phosphate-buffered saline–0.05% Tween 20 solution and incubated 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-human IgG (heavy plus light chains) antibody (Jackson Immunoresearch) (diluted 1:1,000). Reactivity was measured using 100 μl/well of enhanced-chemiluminescence substrate (Amersham Biosciences) and a Fluoroskan Ascent FL fluorometer/luminometer (Thermo Labsystems). The value for the PFD1140-specific signal was calculated by subtracting the value for the control signal (for either the mock translation wells tested with the same serum or the PFD1140w wells tested with nonimmune serum, whichever was greater) and then normalized by dividing it by the value for the signal in the control wells (and as a consequence, the PFD1140w reactivity was expressed as the increase [*n*-fold] over the control value). Differences between groups were analyzed using the Mann-Whitney test.

TABLE 2. Binding phenotypes of parasites from women with pregnancy malaria and from children with malaria

Sample	Gravidity	Result for indicated receptor or test							
		BSA	CD36	CSA	HA	TSP	ICAM	M	Q-PCR
PLCNT 166[a]	Primigravid	−	−	+	−	−	−	+	+
PLCNT 221[a]	Primigravid	−	−	+	−	−	−	ND	+
PLCNT 222[a]	Primigravid	−	−	+	−	−	−	+	ND
PLCNT 918[a]	Primigravid	−	−	+	−	−	−	+	+
PLCNT 920[a]	Primigravid	−	−	+	−	−	−	ND	+
PLCNT 1010[a]	Primigravid	−	−	+	−	−	−	ND	ND
PLCNT 986[a]	Secundigravid	−	−	+	−	−	−	ND	ND
PLCNT 038[a]	Multigravid	−	−	+	−	−	−	+	ND
PLCNT 661[a]	Multigravid	−	−	+	−	−	−	+	+
PLCNT 836[a]	Multigravid	−	−	+	−	−	−	ND	+
CH-TR 095[a]	NA	−	+	−		−	−	+	+
CH-TR 662[a]	NA	−	−	−	−	−	−	+	+
CH-TR 669[a]	NA	−	−	−	−	−	−	+	+
CH-TR 852[a]	NA	−	+	−	−	+	−	+	+
MT-PER 918[b]	Primigravid	−	−	+	−	−	−	+	ND
MT-PER 920[b]	Primigravid	−	−	−	−	−	−	+	+
MT-PER 1000[b]	Primigravid	−	−	−	−	−	−	ND	+
MT-PER 1010[b]	Primigravid	−	−	−	−	−	−	ND	+
MT-PER 986[b]	Secundigravid	−	−	+	−	−	−	ND	+
MT-PER 836[b]	Multigravid	−	−	+	−	−	−	+	+
CH-PER 073[b]	NA							+	+
CH-PER 135[b]	NA	−	+	−	−	−	−	+	+
CH-PER 140[b]	NA	−	−	−	−	+	+	+	ND
CH-PER 372[b]	NA							+	+
CH-PER 413[b]	NA	−	+	−	−	−	+	+	+
CH-PER 425[b]	NA							+	+
CH-PER 451[b]	NA							+	ND
CH-PER 711[b]	NA	−	+	−	−	+	−	+	+

[a] Binding phenotypes of placental parasites from women with pregnancy malaria and of trophozoites cultured from peripheral blood samples drawn from children with malaria. Parasites were extracted directly from infected placentas and tested for interaction with a panel of host receptors by using standard binding assays (16). + indicates ≥20 adherent infected red blood cells in 20 high-power fields for the control (BSA) and test (CD36, CSA, HA, TSP, and ICAM) receptors. Samples denoted with the PLCNT prefix are maternal placental parasites, and those with the CH-TR prefix are trophozoites cultured from infected children. ND, not done; NA, not applicable. Column M indicates samples that were used for microarrays. The transcriptional profiles of placental parasites and children's trophozoites were compared by microarray analysis and validated by Q-PCR. Additional samples were analyzed by Q-PCR for validation of microarray results. PLCNT 986 and PLCNT 1010 are placental samples from the same mothers as peripheral samples used only for Q-PCR validation.

[b] Binding phenotype from peripheral parasites cultured to the trophozoite stage from women with pregnancy malaria and children with clinically diagnosed malaria. Samples denoted with the MT-PER prefix are from maternal peripheral parasites, and those with the CH-PER prefix are from peripheral parasites from children. As described in footnote *a*, the transcriptional profiles of peripheral parasites from women with pregnancy malaria and children with malaria were compared by microarray analysis and validated by Q-PCR.

RESULTS

Binding characteristics of clinical samples of parasites. The receptor binding profiles of parasite samples were determined by standard static adhesion assays (16). Parasites obtained from placentas were assessed directly for their binding properties without prior in vitro cultivation. Placental IEs bound CSA but not other receptors, including HA (Table 2). Trophozoites derived in vitro from circulating peripheral parasites of both pregnant women and children were assayed for binding against the same receptors. Parasites cultured from maternal peripheral blood samples also adhered exclusively to CSA (Table 2). In contrast, parasites collected from children did not bind to CSA but instead adhered to a variety of receptors, including CD36, indicating the more diverse array of parasite forms infecting children (Table 2). No binding was detected for samples where parasitemia was low, consistent with other receptor binding studies (16).

Developmental stage of clinical samples defined by microarray analysis. Genome-wide transcript expression profiles, taken at hourly intervals of the 48-h intraerythrocytic developmental cycle of HB3 parasites, have revealed a pattern of periodic expression for over 80% of the expressed genes (8, 39). The phaseogram depicting gene expression for the complete HB3 intraerythrocytic cycle (40) emphasizes that cell cycle timing is an important potential confounding factor in studies that examine phenotype-specific gene expression. We used whole-genome spotted microarrays to obtain the gene expression patterns of clinical parasite samples, studied immediately after collection or during the first cycle of in vitro development (Table 2). To map the cell cycle position for each sample, the expression profile obtained by microarray analysis was compared to the publicly available HB3 intraerythrocytic cycle time series (DeRisi laboratory malaria transcriptome database [http://malaria.ucsf.edu]). The HB3 time point with the closest resemblance to each clinical sample was identified using both Euclidean distance measurements and correlation comparisons between matched probes. The timing of all clinical samples is summarized in Table 3. The cell cycle position of each clinical sample calculated by the two methods was highly concordant, adding a measure of confidence to our analyses.

These measurements indicate that the peripheral samples are well matched with respect to cell cycle timing, minimizing

TABLE 3. Summary of cell cycle positions of microarray samples as determined by Euclidean distance and correlation distance comparison with the 48-h asexual stage transcriptome of *P. falciparum*

Clinical sample	Closest time point (h) for:	
	Euclidean distance	Correlation distance
MT-PER 836[a]	13	13
MT-PER 918[a]	13	12
MT-PER 920[a]	13	13
CH-PER 073[a]	7	12
CH-PER 135[a]	10	10
CH-PER 140[a]	12	12
CH-PER 372[a]	16	13
CH-PER 413[a]	12	12
CH-PER 425[a]	13	13
CH-PER 451[a]	13	13
CH-PER 711[a]	12	10
PLCNT 038[b]	31	31
PLCNT 166[b]	24	24
PLCNT 222[b]	31	31
PLCNT 661[b]	24	24
PLCNT 918[b]	31	31
CH-TR 095[b]	31	31
CH-TR 662[b]	47	47
CH-TR 669[b]	31	31
CH-TR 852[b]	31	31

[a] Cell cycle timing of peripheral parasites isolated from women with pregnancy malaria and children with malaria that were used to identify genes that are differentially expressed during the ring stage of development (the stage immediately preceding parasite adhesion to the placenta). The HB3 *P. falciparum* transcriptome database (DeRisi laboratory) was used to map the time after invasion of red blood cells for all clinical samples used for microarray analysis.

[b] Cell cycle timing of placental parasites and trophozoites cultured from children used for comparison of gene expression levels for placental binding and nonbinding parasites.

this as a major confounder in our analysis of binding phenotype differences between groups. The closest correlation distance for all peripheral samples maps to between 10 and 13 h of the HB3 blood stage cycle, with the majority of samples falling between 12 and 13 h (Fig. 1A). The corresponding overlaid Euclidean distance plots for all peripheral samples show the same trends (Fig. 1C). Correlation distance plots for replicates of all samples are also shown in Fig. S1 in the supplemental material. All Euclidean distance plots are shown in Fig. S2 in the supplemental material. A high level of reproducibility between arrays is seen.

Placental parasite samples and trophozoites cultured from child-derived samples show greater diversity in cell cycle timing than the peripheral parasite samples. The child-derived sample CH-TR 662 is poorly synchronized and most nearly resembles synchronized HB3 parasites at the 47-h time point rather than those at the trophozoite stage (between 18 and 32 h) (see Fig. S1 and S2 in the supplemental material). This sample was excluded from all further analyses. Two out of five placental parasite samples map to 24 h, while all other placental samples, as well as all of the remaining trophozoite samples from children, most closely resemble the 31-h HB3 profile. To visualize these differences, child-derived sample CH-TR 852 with a best fit at 31 h is shown superimposed on the PLCNT 166 correlation plot (Fig. 1B) and the corresponding Euclidean distance plot (Fig. 1D), which maps to 24 h. Correlation and Euclidean distance measurements for the placental and child-derived trophozoite samples indicate that the two methods yield similar results (see Fig. S1 and S2 in the supplemental material). Hierarchical clustering of all time profiles places PLCNT 661 and PLCNT 166 between the ring and trophozoite samples (Fig. 1E). The correlation and Euclidian distance results were consistent with the PCA results in which the 48-h intraerythrocytic developmental cycle is reduced to three principal components corresponding to the early ring-late schizont, late ring-early trophozoite, and late trophozoite-early schizont stages (Fig. 1F).

***var2csa* is highly expressed by peripheral parasites from pregnant women.** We aligned 3D7, IT4, and Dd2 sequences of *var2csa* and identified nine unique, well conserved 70-mer sequences for use as microarray elements (Fig. 2). These elements are also generally conserved in the recently sequenced Ghanaian clinical parasite sample (Fig. 2). Multiple pairwise comparisons between three maternal peripheral samples and eight samples from children were performed (see Table S1 in the supplemental material). Four out of seven *var2csa* elements were well detected by a probe prepared from maternal parasites; two of three elements that performed poorly were the least conserved compared to the Ghanaian sequence. Two additional elements with greater than 90% sequence identity with *var2csa* were discovered during analysis of the hybridization experiments. Element *var2csa* 4038 (derived from a cDNA clone of the placental parasite pl28b) was originally thought to be a divergent DBLγ domain of *varIcsa* (19) but is identified here as a clinical variant of *var2csa*. The other element maps to the probable 5′ untranslated region (37) of *var2csa* 653 bp upstream from the putative initiator methionine (Fig. 2).

Of 396 possible *var* elements spotted on these arrays, the probe prepared from pregnancy malaria peripheral parasites hybridized exclusively to 11 *var2csa* elements (Fig. 3A). In contrast, peripheral parasites from children showed no unifying trends. Signals were reliably detected for 56 elements that have homology to 30 genes and pseudogenes in the 3D7 genome. Elements designed from conserved regions of group A PfEMP1 genes were most frequently recognized by parasites from children, and some minor trends can be observed. For example, four *PFD1235w* elements, positioned along 1,850 bp of the gene, were recognized by CH-PER 711, consistent with a high degree of conservation. Signals from three out of four elements of *PFF0020c* were also detected for CH-PER 711. In seven other cases, two elements from the same 3D7 gene hybridized with the same clinical sample. Interestingly, there is very little consistent recognition of a particular element across samples from children (Fig. 3B). The diversity of *var* transcripts detected in parasites from children could be due in part to differences in binding phenotype profiles between parasites. Alternatively, CD36 binding is common to most of these samples from children, and multiple 3D7 *var* genes appear to have the capacity to bind CD36 in vitro (55), which may also be the case in vivo.

Six up-regulated genes distinguish pregnancy malaria parasites from child-derived parasites at both the ring and trophozoite stages. Multiple regression analysis of all sample replicates was performed, and an average value was extracted for each element. Hierarchical clustering of the Euclidean averaged distances produced a distinct cluster that included multiple *var2csa* elements and elements from eight genes (Fig.

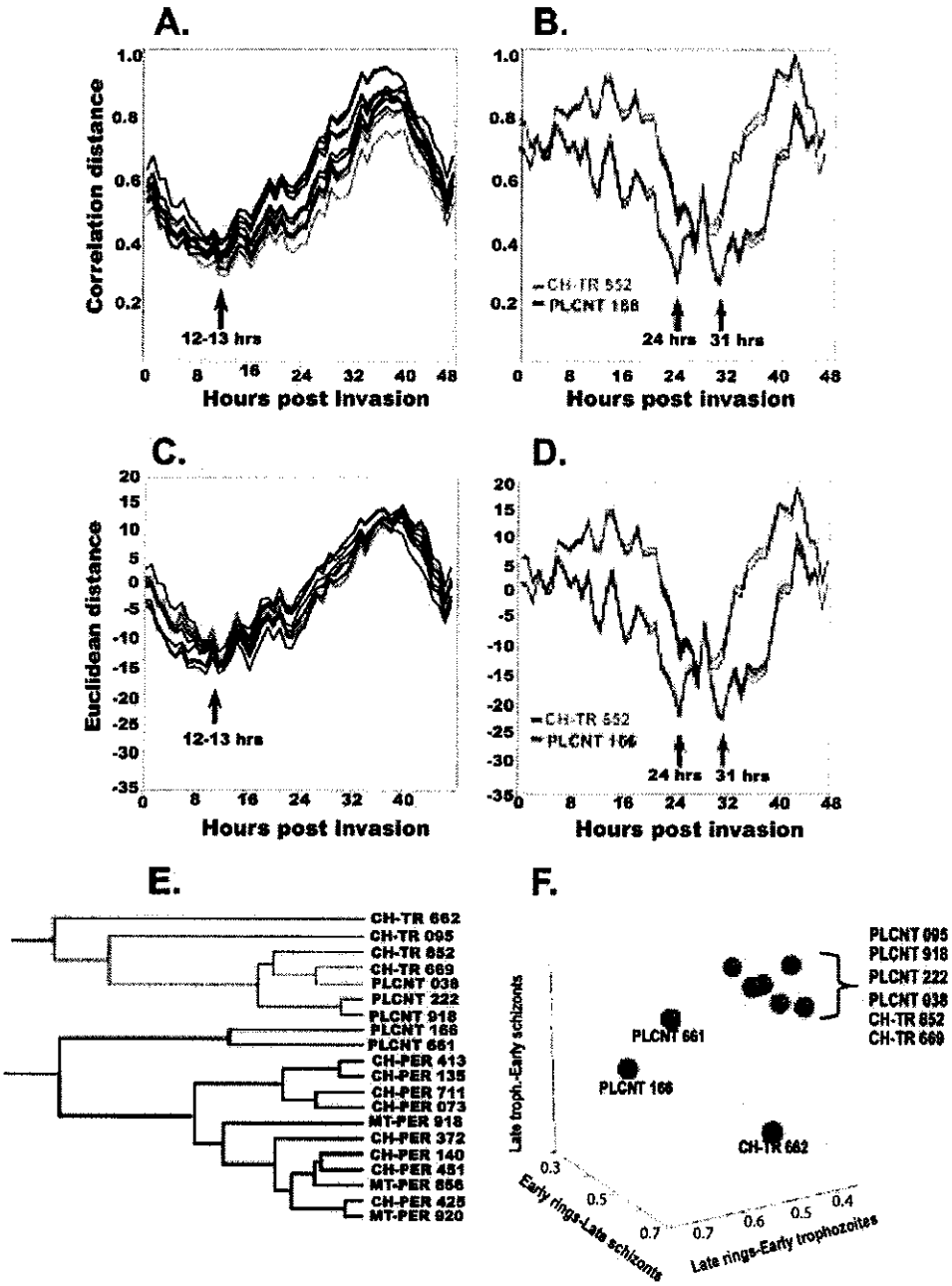

FIG. 1. Timing analysis of each clinical sample in the 48-h intraerythrocytic cycle. (A) Correlation distance plots comparing averages for replicates for each peripheral blood parasite sample with the 51 time points from the 48-h HB3 transcriptional profile analysis (8). The three peripheral samples from women with pregnancy malaria are shown in green, and eight peripheral samples from children with malaria are shown in black. (B) Correlation distance plot comparing averages for replicate samples of placental parasite PLCNT 166 and averages for replicates of CH TR 852. (C and D) Euclidean distance plots for samples in panels A and B, respectively. (E). Hierarchical clustering of correlation time profiles of all clinical samples. (F). Placental trophozoite samples and cultured trophozoite samples from children were loaded onto three principal-component coordinates to compare their positions in the cell cycle. Child-derived trophozoite samples CH-TR 095, CH-TR 852, and CH-TR 669 and placental samples PLCNT 038, PLCNT 222, and PLCNT 918 are comparably positioned (shown in blue). Placental samples PLCNT166 and PLCNT 661 have greater representation in the late ring-early trophozoite component (shown in red). Sample CH-TR 662 (in red) has a distinct timing profile. The 31-h cluster is shown in blue. Samples are identified left to right.

4A). All were expressed eightfold or higher in maternal peripheral parasites compared to what was found in peripheral parasites from children. *PFB0115w* was expressed 47.5-fold higher in maternal parasites, a magnitude of differential expression comparable to that of the best-recognized elements of *var2csa*. Q-PCR of peripheral parasite samples from women with pregnancy malaria (including some samples that were and some that were not used for microarray studies) confirmed that

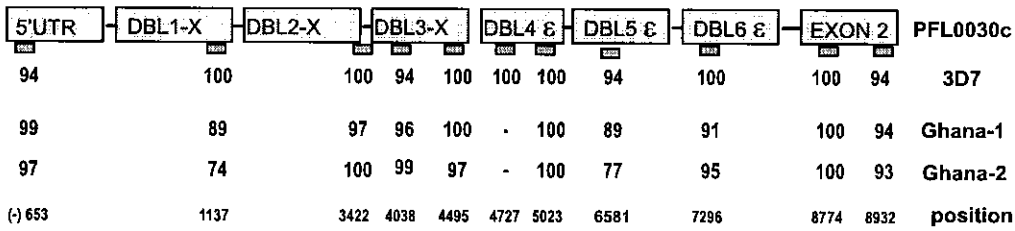

FIG. 2. *var2csa* microarray elements. Schematic of consensus 70-mer nucleotide microarray elements showing DBL domain assignments. Elements were designed by aligning *var2csa* sequences from different laboratory strains of *P. falciparum*. 3D7, Ghana-1, and Ghana-2 indicate percent sequence identity of array elements with 3D7 and two recently sequenced *var2csa* homologs obtained from a clinical sample (Ghanaian *P. falciparum* genome sequence; Sanger Center). Operon element M59463_1 (made against *MAL13P1.353*, no longer annotated) maps to bp −653 relative to the *PFL0030c* translation start site. The element at position 4038 was designed from a DNA sequence amplified from placental isolate pl 28b, using degenerate DBLγ domain primers (19). Element positions are relative to the 3D7 gene *PFL0030c*. UTR, untranslated region.

these genes were significantly more highly expressed by parasites from maternal peripheral blood samples (Fig. 4B).

As was seen for the peripheral parasite microarrays (see Table S1 in the supplemental material), the number of genes that were expressed twofold or higher by placental parasites is small (see Table S2 in the supplemental material). Among the genes that were upregulated in peripheral parasites of pregnant women, the expression levels of *PFB0115w*, *PFD1140w*, *PFI1785w*, *PFL0050c*, *MAL13P1.320*, and *var2csa* were also upregulated in placental parasites, whereas those of *MAL13P1.470*, *PFF0435w*, and *PFL1385c* were not (Fig. 4C). Genes found by microarray analysis to have increased expression in placental parasites were validated by Q-PCR (Fig. 4D).

In total, 8 different maternal samples derived from either peripheral blood samples or placentas from women with pregnancy malaria were compared with 11 parasite samples collected from peripheral blood samples from children and assayed directly or cultured to the trophozoite stage. A small number of genes were found to be expressed at higher levels by parasites from women with pregnancy malaria. Despite differences in the developmental stages of maternal parasites, six genes were common to both analyses. In addition to these six genes, *MAL7P1.225* and *PF10_0013* were more highly expressed by placental parasites, while *MAL13P1.470* was more highly expressed by ring stage parasites from pregnant women.

Genes up-regulated in pregnancy malaria parasites have coordinated timing of expression. The transcription profiles for genes that differ most consistently over the course of blood stage development were extracted from the intraerythrocytic cycle database (malaria transcriptome database [http://malaria.ucsf.edu]). K means clustering produced six expression profiles (Fig. 5). Four of six clusters have well-defined expression patterns roughly corresponding to peaks at the ring stage, late ring/early trophozoite stage, mid- to late trophozoite stage, and schizont/early ring stage (Fig. 5A, B, C, and D). One cluster is less well defined and has broader transcript timing (Fig. 5E), and one cluster is composed of genes whose expression levels are close to the background level across the intraerythrocytic cycle (Fig. 5F).

The genes (*PFB0115w*, *PFD1140w*, *PFI1785w*, and *PFL0050c*) that were most highly expressed in both peripheral and placental parasites from pregnant women cluster with genes expressed maximally at the late ring/early trophozoite stage (Fig. 5A). This expression pattern coincides with the disappearance of trophozoite stage parasites from the peripheral circulation because of sequestration in tissues. Expression of *var2csa* was not detected in the HB3 time series; maximal *var2csa* expression was observed at 6 hours postinfection in the 3D7 blood stage time course (malaria transcriptome database [http://malaria.ucsf.edu]). Other studies indicate that the *var2csa* message is present during the ring and early trophozoite stages (60). *MAL13P1.320* does not follow the same transcription profile and instead clusters with genes expressed at a low level across much of the HB3 cell cycle (Fig. 5F).

Serum recognition by PFD1140w is gender and parity specific. Genes with increased transcription in parasites from pregnant women were expressed by in vitro translation and tested by an enzyme-linked immunosorbent assay for reactivity against East African sera. Thirty-three sera from multigravid and 20 sera from primigravid women from a high-malaria-transmission area in Tanzania were compared for reactivity against *PFD1140w*, *PFI1785w*, *PFB0115w*, *MAL13P1.320*, and *MAL13P1.470*. Reactivity against recombinant PFD1140w was significantly higher with sera from multigravid women, suggesting that antibodies are acquired over successive pregnancies (Fig. 6). None of the other expressed proteins showed significant parity-specific seroreactivity profiles. PFB0115w was more reactive against sera from multigravid women, but differences between multigravid and primigravid sera were not statistically significant. Other expressed proteins were poorly recognized by sera, possibly because of problems with protein folding (data not shown).

In rural areas around towns such as Muheza, Tanzania, most women have their first pregnancy within a short age window, and therefore it is generally difficult to age match primigravidae with multigravidae. To eliminate age as a confounder, we compared the reactivities of sera from 22 multigravid women (median age, 28 years) with those of sera from 22 males (median age, 29 years) living in an area of intense malaria transmission near Kisumu, Kenya. Sera from multigravid women were significantly more reactive than male sera (Fig. 6), indicating that differences in reactivity were not related to age but were related to pregnancy and parity. The seroreactivity profile shown for PFD1140w matches that previously reported for VAR2CSA using the same sera (50). Reactivity to the control malaria antigen apical membrane antigen (AMA-1) did not differ significantly between primigravidae and multigravidae in Tanzania

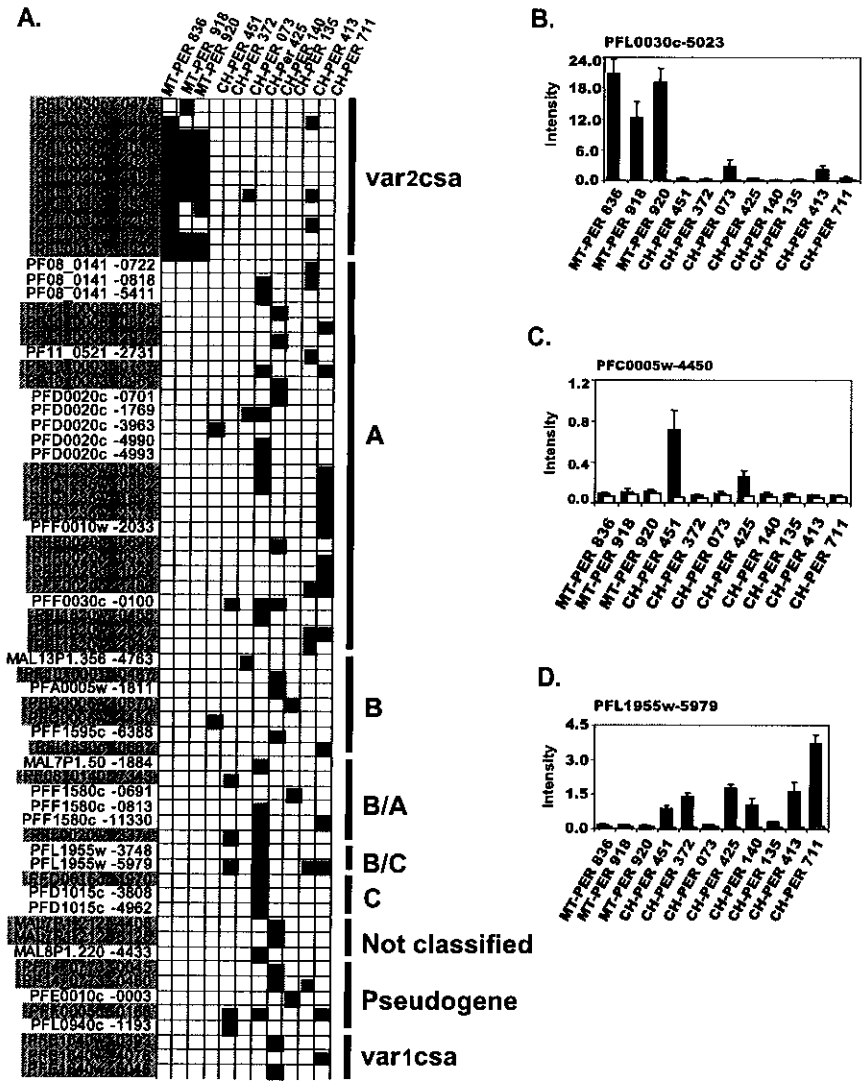

FIG. 3. PfEMP1 gene expression. (A) Checkerboard representation of expression by members of the PfEMP1 gene family by different clinical samples. Gray boxes denote samples that hybridized to that PfEMP1 probe. PfEMP1 genes were grouped according to the classification scheme in reference 37. The probe position for each PfEMP1 element is based on the 3D7 nucleotide sequence and is indicated as part of each gene name. (B, C, D) Comparison of signal intensities (filled bars) among clinical samples for elements PFL0030c-5053 (B), PFC0005w-4450 (C), and PFL1955w-5979 (D). Open bars show background intensity.

or between males and multigravidae in Kenya, and all East African sera reacted to this antigen in our studies (50).

DISCUSSION

The receptor binding profiles of parasites derived from pregnant women did not overlap with those for children (Table 2), thus providing an opportunity to identify differentially regulated genes that distinguish placental parasites from other parasite forms.

Since *var* gene transcription is greatest during ring stage development (36), we speculated that other adhesion proteins or proteins related to the binding phenotype might have coordinated expression in ring stage parasites. We assumed that peripheral parasites were representative of placental parasites, because peripheral parasites of pregnant women mature into CSA-binding trophozoites (16, 17) and develop seroreactivity profiles similar to those of placental parasites (49). Consistent with this, we found a striking overlap in up-regulated genes from both peripheral and placental parasites (Table 4).

Cell cycle analysis. With the exception of child-derived trophozoite parasites that had been grown in culture overnight, all clinical samples used in these studies were processed immediately after collection for subsequent use in transcriptional studies. To determine the cell cycle timing for each clinical sample used in this study, the transcriptional profile obtained by microarray analysis was compared with the transcriptional profile at every hour of the 48-h intraerythrocytic cycle, using the publicly available data set (malaria transcriptome database [http://malaria.ucsf.edu]), by Euclidean and correlation distance measurements. Similar results were obtained using both

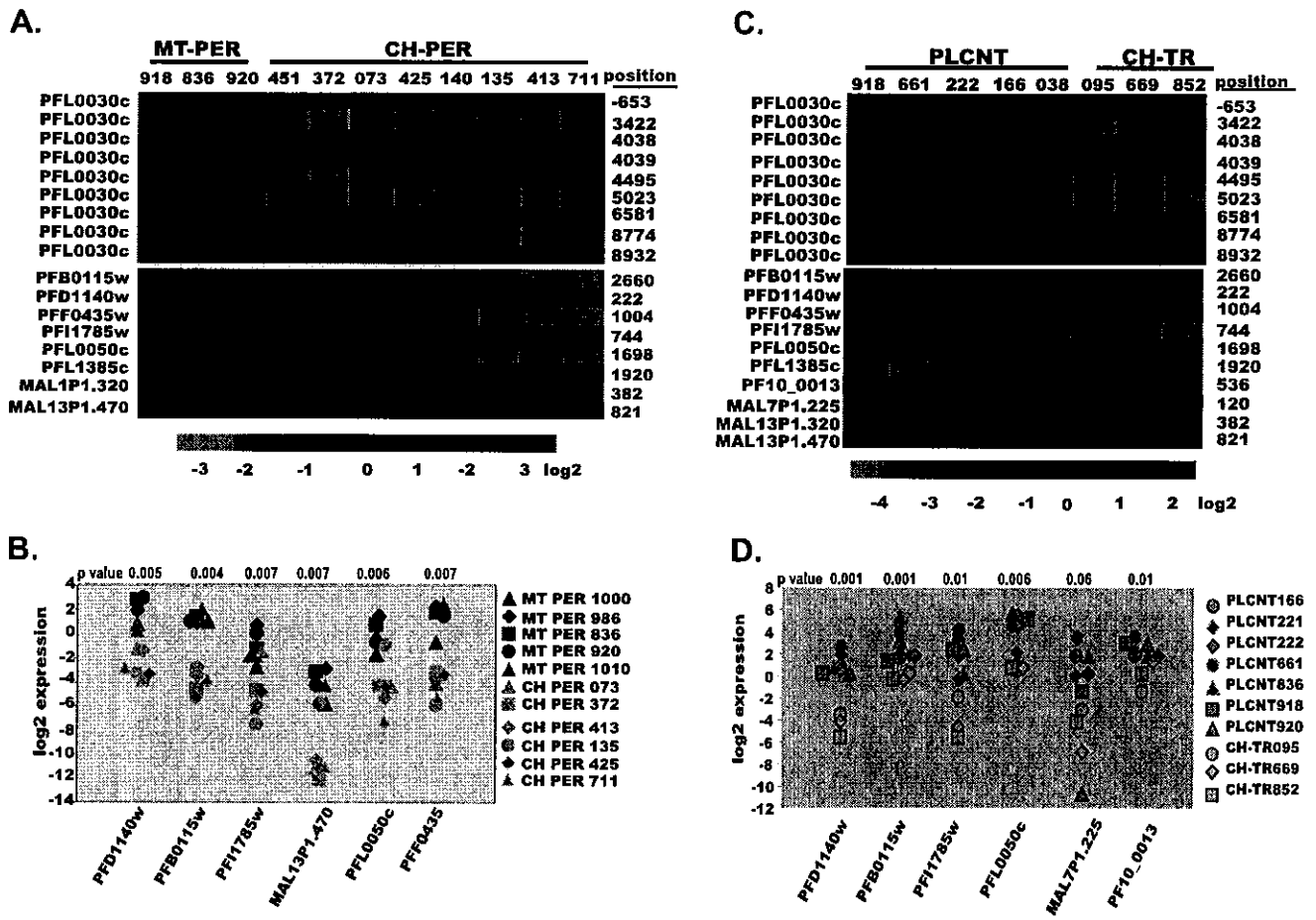

FIG. 4. Genes with increased expression in parasites obtained from women with pregnancy malaria. (A) Heat map of highly up-regulated genes expressed by peripheral parasites from women with pregnancy malaria based on Euclidean hierarchical clustering. (B) Expression levels of up-regulated genes identified by microarray analysis compared to seryl tRNA synthase constitutive control levels as detected by Q-PCR. (C) Heat map of transcription by trophozoite stage parasites, focused on the *var2csa* gene cluster identified in maternal peripheral parasites. (D) Q-PCR analysis of trophozoite stage gene expression.

methods (Fig. 1). We found that ring stage parasites from peripheral blood samples matched HB3 timing most closely at 10 to 13 h after invasion of the red blood cell. The basis for the striking concordance in cell cycle timing of peripheral parasites is unknown but may be due in part to the increased level of transcription in late-ring-stage (10 to 13 h) parasites, which could contribute disproportionately to the hybridization signal. Regardless, the closely matched cell cycle timing gives us greater confidence that expression differences between these parasite phenotypes are related to CSA binding or to the specialized physiological milieu of the placenta rather than to differences in the cell cycle.

In contrast to the peripheral parasites analyzed here, the trophozoite stage samples fall into two distinct time classes at 24 h and 31 h of development. When the 31-h placental samples (PLCNT 038, PLCNT 222, and PLCNT 918) are compared to 31-h child-derived trophozoite samples (CH-TR 095 and CH-TR 852), a subset of the genes identified in the ring stage comparisons (*PFD1140w*, *PFB0115w*, *PFI1785w*, *PFL0050c* and *PFL0030c*/*var2csa*) are found to be expressed at two- to fourfold-higher levels in placental parasites (see Table S2 in the supplemental material). Comparison of the 24-h placental parasite samples (PLCNT 661 and PLCNT 166) with the 31-h trophozoites from children suggests a much higher level of differential expression in these genes (see Table S2 in the supplemental material), presumably due in part to the differences in cell cycle timing. This emphasizes the importance of determining the developmental stage of parasites in clinical samples.

***var2csa* is up-regulated in all parasite samples from women with pregnancy malaria.** VAR2CSA is a semiconserved member of the PfEMP1 family (34, 60) that has been previously implicated as a mediator of adhesion to the placental receptor CSA. We found that *var2csa* was differentially expressed by both peripheral and placental binding parasites of pregnant women. In contrast, *var2csa* expression was reliably detected in only one child-derived sample (CH-PER 413), and here, only a few elements were hybridized and the signal was much less intense than those in maternal samples (Fig. 3B). The *var2csa* hybridization pattern of CH-PER 413 may have resulted from cross-hybridization with another expressed PfEMP1; however, this was not seen for other samples from children. Alternatively, the sequence of *var2csa* expressed by this parasite may

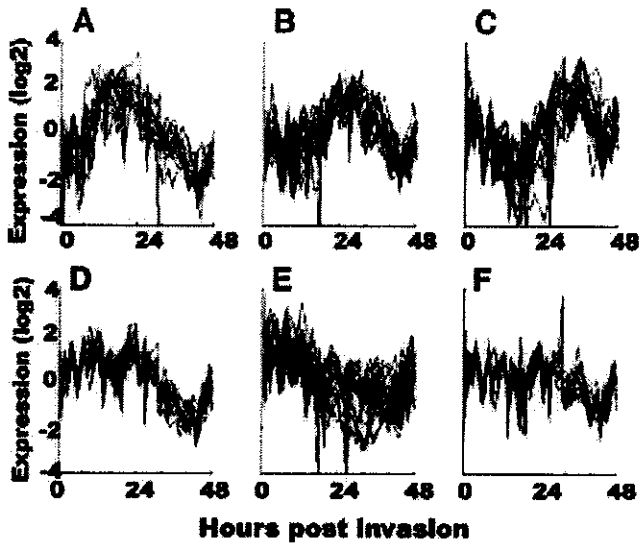

FIG. 5. Coordinated expression of genes with increased expression in parasites from pregnant women. (A to F) In microarray data from reference 8, K means clustering was used to define six major expression profiles of genes during intraerythrocytic development of laboratory parasites. Four genes that are up-regulated in parasites from pregnant women (*PFB0115w*, *PFD1140w*, *PFI1785w*, and *PFL0050c*) (highlighted in red) cluster with a single group of genes that are most highly expressed during late-ring/early-trophozoite development.

diverge extensively from the sequence of *var2csa* expressed by parasites from pregnant women. Curiously, two elements recognized by the CH-PER 413 probe appear to be the most variant of those designed from conserved regions of this gene. This may indicate that a subset of *var2csa* variants is specifically associated with placental parasites.

Five genes are coexpressed with *var2csa* by parasites causing pregnancy malaria. We identified a small cohort of genes that mirror the *var2csa* expression profile in both peripheral and placental parasites from pregnant women. Genes included in this group predict proteins with transmembrane and/or signal sequences (Table 4). Three of the five differentially expressed genes identified here have putative export motifs (Table 4) that are consistent with transit to the erythrocyte (30, 44). *PFD1140w* has a PEXEL sequence (44), while *PFL0050c* and *PFI1785w* each have a VTS motif (30). Interestingly, *PFI1785w* belongs to a *P. falciparum* family composed of 16 uncharacterized hypothetical genes, most of which have predicted export sequences (PlasmoDB). *PFB0115w* and *MAL13P1.320* have neither a PEXEL nor a VTS motif. However, this does not exclude trafficking of these proteins to the red blood cell by alternate export pathways, as has been suggested for skeleton binding protein 1 (*sbp1* protein) and Maurer's cleft histidine-rich protein, which lack PEXEL/VTS motifs but nevertheless localize to the Maurer's clefts in the red blood cell cytoplasm.

Additional genes were differentially expressed by only ring stage or only mature-stage parasites from pregnant women and may play a role in the placental parasite phenotype as well. These genes include two members of the HISTa gene family (61), consisting of 42 paralogs in *P. falciparum* (*P. falciparum* gene database, Sanger Center [http://www.genedb.org/]) that encode proteins with putative PEXEL sequences (61) as well as *PF10_0013*, which has a PEXEL motif (44) and has been localized to the Maurer's cleft by proteomic analysis (68) (Table 4). Future studies will be required to determine whether these proteins are trafficked to the erythrocyte as well as how these expanded gene families affect the pathogenesis of *P. falciparum* malaria. A recent photolabeling study suggested that a 22-kDa protein on the IE surface may bind CSA (26).

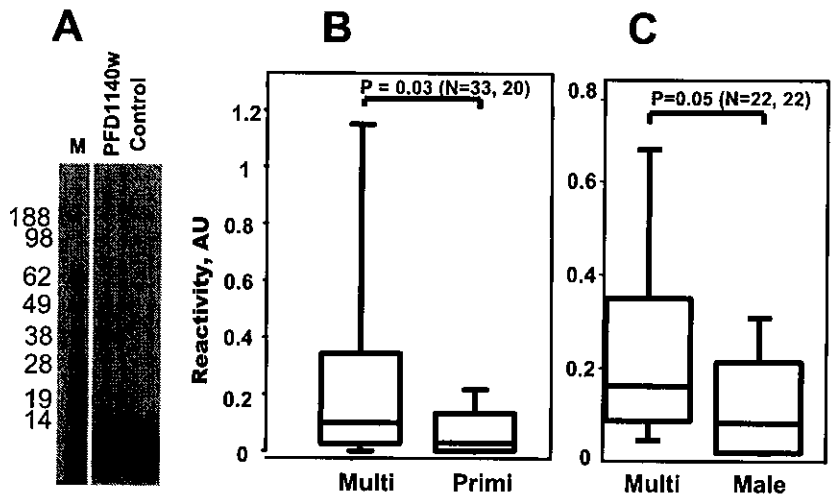

FIG. 6. PFD1140w seroreactivity. Recombinant PFD1140w is recognized by human sera from areas of malaria endemicity in a gender- and parity-dependent manner. (A) Coomassie-stained gel of affinity-purified cell-free expressed PFD1140w. Control, material purified from mock-translated reaction mixture. M, molecular mass markers with corresponding values (in kilodaltons). Low-molecular-mass material in both samples is FLAG peptide used for elution. (B) Reactivity of PFD1140w with sera from Muheza, Tanzania, where malaria transmission is intense. Multi, sera from multigravid women. Primi, sera from primigravid women. AU, arbitrary units. *P*, Mann-Whitney *P* value. *N* indicates the number of samples. The top of the box, bottom of the box, and line through the middle of the box correspond to the 75th, 25th, and 50th (median) percentiles, respectively. The whiskers indicate the 10th and 90th percentiles. (C) Reactivity of PFD1140w with sera from Kenya, where malaria transmission is intense. Male, sera from male donors. Other abbreviations and representations are as described for panel B.

TABLE 4. Characteristics of genes with increased expression in pregnancy malaria parasites by microarray analysis

Gene	Fold increase in expression of:		Result for:			Potential export sequence[e]	Mass (kDa)	Maximum expression time (h)[f]	Stage(s) of expression[g]	No. of paralogs	Function
	Maternal peripheral parasites	Placental parasites	SS/TM[a]	VTS	PEXEL						
PFL0030c	14.2	6.4	−/+[b]	+	+	RWLTE	355.2	ND	ND	59	PfEMP1
PFB0115w	47.5	4.0	−/−[c]	−	−		141.8	16	ET,LT	1	Hypothetical
PFD1140w	16.9	6.3	+/+[b]	−	+	RNLSE	40.8	18	LR,ET,LT	3	Hypothetical
MAL13P1.320	16.9	3.4	+/+	−	−		80.1	ND	LR,ET,LT	1	Hypothetical
PFL0050c	9.6	6.2	−/−[c]	+	−	RILSS	77.1	18	ES	1	Hypothetical
PFI1785W	8.2	10.3	−/−[c]	+	−	RISLE	39.5	22	ET,LT	17	Hypothetical
PF10_0013	NI	4.4	+/−	−	+	RLISE	27.6	18	LR	4	Hypothetical
MAL13P1.470	9.9	NI	−/+[b]	−	−	RNLSE	48.9	20	NP	42	Hypothetical
MAL7P1.225	0.6	5.1	−/+[b,d]	−	−	RNLSE	29.9	18	NP	42	Hypothetical

[a] The transmembrane domain (TM) was predicted by transmembrane prediction alogrithm TMHMM unless otherwise stated. SS, signal sequence.
[b] Potential signal anchor sequence.
[c] TM was predicted by alternate transmembrane prediction algorithms TMAP, TMPRED, and/or TOPPRED2.
[d] Alternate annotation, chr 7 glimmerm_35.
[e] Predicted by PlasmoDB 5.2.
[f] For a synchronized HB3 time series profile (8).
[g] 3D7 expression time course (38). LR, late ring; ET, early trophozoite; LT, late trophozoite; ES, early schizont; NP, not probed; ND, not detected; NI, not increased.

None of the genes identified here predict a protein of this size, although PF10_0013 (27.6 kDa) and MAL7P1.225 (29.9 kDa) predict slightly larger molecules.

Genes identified in the placental malaria cohort have been linked to adhesion phenotypes in two other microarray studies. Mok and coworkers found *var2csa* to be one of three *var* genes expressed at higher levels by rosetting parasites than by CD36-selected 3D7 parasites (45). This is curious because rosetting parasites are common in children, where *var2csa* transcription is atypical, but rarely observed in placental isolates, where *var2csa* transcription is generally detected (14, 56). Furthermore, the parasites selected to form rosettes failed to bind CSA (although they did bind to placental sections in a CSA-independent manner). Notably, *PFB0115w* was also differentially expressed by the rosetting parasites, suggesting that expression of *var2csa* and *PFB0115w* may be linked, as was seen in our study.

An earlier study used microarrays to compare FCR3 parasites selected to bind either CSA or CD36 (52). The microarrays included FCR3 *var*-specific probes to allow detection of PfEMP1 genes. CD36-selected cells differentially expressed a number of *var* genes, while only *var2csa* was differentially expressed by CSA-selected parasites. Many non-*var* genes were also found to have increased expression by FCR3 CSA-binding parasites, including *PFB0115w*, *PFD1140w*, and *MAL13P1.470*, which were among the most highly differentially expressed genes at both the ring and trophozoite stages (52). In contrast to the FCR3 study, we found only six genes with increased expression in all maternal parasite samples. The smaller cohort generated by our study might result because more samples were compared (8 maternal samples were compared to 11 samples from children) or because our studies used clinical samples that may better discriminate relevant genes. In the FCR3 study, two biological replicates were analyzed at the ring and trophozoite stages. The overlap in gene expression between parasites from women with pregnancy malaria and CSA-selected FCR3 parasites suggests that the genes identified here as belonging to the pregnancy malaria cohort merit further investigation. Our results suggest that only a few differentially expressed genes, including *var2csa*, distinguish placental parasites. However, due to sequence variation, we cannot exclude roles for highly polymorphic genes, like rifins, stevors, Pfmc-2TM, and other PfEMP1 genes, in the development of the placental parasite phenotype (9).

Seroreactivity of PFD1140w is gender and parity specific. VAR2CSA has been previously shown to have gender- and parity-specific recognition by immune serum that corresponds to the pattern of acquired immunity to pregnancy malaria (59, 67). PFD1140w showed a similar seroreactivity profile (Fig. 6). Whether antibodies against PFD1140w impair or block adhesion to CSA or have some other functional activity against placental parasites will be addressed in future studies. PFB0115w had higher reactivity with multigravid sera than with male sera, but these differences were not significant. PFB0115w should be studied in larger seroepidemiology surveys to assess whether this trend reflects a real difference in seroreactivity.

In conclusion, our results indicate that a small suite of genes is associated with the development of the placental parasite phenotype. Although the roles of the corresponding proteins in malaria pathogenesis or immunity remain unclear, we speculate that some of these proteins may have functions to interface with the pregnant host in the specialized milieu of the placenta, such as the putative role in which VAR2CSA adheres to CSA. Any proteins that localize to the surface of the IE will become important candidates for a pregnancy malaria vaccine, particularly in view of their highly conserved sequences. Defining the separate roles of these genes in the development of placental parasites could lead to a better understanding of mechanisms by which parasites acquire distinct binding properties and yield novel targets for intervention.

ACKNOWLEDGMENTS

We thank Chris Ramsborg for critical reading of the manuscript; Wonjong Moon, Atis Muehlenbachs, Jay Gerlach, and Paul Shannon for help with data analysis; Jeff Dorfman for helpful discussions; Lori

Anderson for technical assistance; Sue Kraemer, Amy Springer, and Joe Smith for providing advice and sharing unpublished data; JinLong Li for IT support; Maximillian Mpina, Martin Mhando, and Dalley Andeoli for performing binding assays; and the MOMS Project nurses stationed at Muheza DDH who collected and processed the samples used in this study.

This work was supported by the U.S. National Institutes of Health (grant AI52059), the Bill and Melinda Gates Foundation (grant 29202), and the Grand Challenges in Global Health/Foundation for the NIH (grant 1364).

REFERENCES

1. **Achur, R. N., M. Valiyaveettil, A. Alkhalil, C. F. Ockenhouse, and D. C. Gowda.** 2000. Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of *Plasmodium falciparum*-infected erythrocytes to the placenta. J. Biol. Chem. **275**:40344–40356.
2. **Achur, R. N., M. Valiyaveettil, and D. C. Gowda.** 2003. The low sulfated chondroitin sulfate proteoglycans of human placenta have sulfate group-clustered domains that can efficiently bind *Plasmodium falciparum*-infected erythrocytes. J. Biol. Chem. **278**:11705–11713.
3. **Andrews, K. T., L. A. Pirrit, J. M. Przyborski, C. P. Sanchez, Y. Sterkers, S. Ricken, H. Wickert, C. Lepolard, M. Avril, A. Scherf, J. Gysin, and M. Lanzer.** 2003. Recovery of adhesion to chondroitin-4-sulphate in *Plasmodium falciparum* varCSA disruption mutants by antigenically similar PfEMP1 variants. Mol. Microbiol. **49**:655–669.
4. **Barfod, L., N. L. Bernasconi, M. Dahlback, D. Jarrossay, P. H. Andersen, A. Salanti, M. F. Ofori, L. Turner, M. Resende, M. A. Nielsen, T. G. Theander, F. Sallusto, A. Lanzavecchia, and L. Hviid.** 2007. Human pregnancy-associated malaria-specific B cells target polymorphic, conformational epitopes in VAR2CSA. Mol. Microbiol. **63**:335–347.
5. **Baruch, D. I., J. A. Gormely, C. Ma, R. J. Howard, and B. L. Pasloske.** 1996. *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA **93**:3497–3502.
6. **Beeson, J. G., G. V. Brown, M. E. Molyneux, C. Mhango, F. Dzinjalamala, and S. J. Rogerson.** 1999. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J. Infect. Dis. **180**:464–472.
7. **Botha, M., E. R. Pesce, and G. L. Blatch.** 2007. The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host. Int. J. Biochem. Cell Biol. [Epub ahead of print.] doi:10.1016/j.biocel.2007.02.011.
8. **Bozdech, Z., M. Llinas, B. L. Pulliam, E. D. Wong, J. Zhu, and J. L. DeRisi.** 2003. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. PLoS Biol. **1**:E5.
9. **Bozdech, Z., J. Zhu, M. P. Joachimiak, F. E. Cohen, B. Pulliam, and J. L. DeRisi.** 2003. Expression profiling of the schizont and trophozoite stages of *Plasmodium falciparum* with a long-oligonucleotide microarray. Genome Biol. **4**:R9.
10. **Buffet, P. A., B. Gamain, C. Scheidig, D. Baruch, J. D. Smith, R. Hernandez-Rivas, B. Pouvelle, S. Oishi, N. Fujii, T. Fusai, D. Parzy, L. H. Miller, J. Gysin, and A. Scherf.** 1999. *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. Proc. Natl. Acad. Sci. USA **96**:12743–12748.
11. **Bull, P. C., B. S. Lowe, M. Kortok, C. S. Molyneux, C. I. Newbold, and K. Marsh.** 1998. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat. Med. **4**:358–360.
12. **Cooke, B. M., D. W. Buckingham, F. K. Glenister, K. M. Fernandez, L. H. Bannister, M. Marti, N. Mohandas, and R. L. Coppel.** 2006. A Maurer's cleft-associated protein is essential for expression of the major malaria virulence antigen on the surface of infected red blood cells. J. Cell Biol. **172**:899–908.
13. **Duffy, M. F., T. J. Byrne, S. R. Elliott, D. W. Wilson, S. J. Rogerson, J. G. Beeson, R. Noviyanti, and G. V. Brown.** 2005. Broad analysis reveals a consistent pattern of var gene transcription in *Plasmodium falciparum* repeatedly selected for a defined adhesion phenotype. Mol. Microbiol. **56**:774–788.
14. **Duffy, M. F., A. Caragounis, R. Noviyanti, H. M. Kyriacou, E. K. Choong, K. Boysen, J. Healer, J. A. Rowe, M. E. Molyneux, G. V. Brown, and S. J. Rogerson.** 2006. Transcribed *var* genes associated with placental malaria in Malawian women. Infect. Immun. **74**:4875–4883.
15. **Elliott, S. R., M. F. Duffy, T. J. Byrne, J. G. Beeson, E. J. Mann, D. W. Wilson, S. J. Rogerson, and G. V. Brown.** 2005. Cross-reactive surface epitopes on chondroitin sulfate A-adherent *Plasmodium falciparum*-infected erythrocytes are associated with transcription of *var2csa*. Infect. Immun. **73**:2848–2856.
16. **Fried, M., G. J. Domingo, C. D. Gowda, T. K. Mutabingwa, and P. E. Duffy.** 2006. *Plasmodium falciparum*: chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta. Exp. Parasitol. **113**:36–42.
17. **Fried, M., and P. E. Duffy.** 1996. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science **272**:1502–1504.
18. **Fried, M., and P. E. Duffy.** 1998. Maternal malaria and parasite adhesion. J. Mol. Med. **76**:162–171.
19. **Fried, M., and P. E. Duffy.** 2002. Two DBLgamma subtypes are commonly expressed by placental isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **122**:201–210.
20. **Fried, M., R. M. Lauder, and P. E. Duffy.** 2000. *Plasmodium falciparum*: adhesion of placental isolates modulated by the sulfation characteristics of the glycosaminoglycan receptor. Exp. Parasitol. **95**:75–78.
21. **Fried, M., F. Nosten, A. Brockman, B. J. Brabin, and P. E. Duffy.** 1998. Maternal antibodies block malaria. Nature **395**:851–852.
22. **Gamain, B., S. Gratepanche, L. H. Miller, and D. I. Baruch.** 2002. Molecular basis for the dichotomy in *Plasmodium falciparum* adhesion to CD36 and chondroitin sulfate A. Proc. Natl. Acad. Sci. USA **99**:10020–10024.
23. **Gamain, B., A. R. Trimnell, C. Scheidig, A. Scherf, L. H. Miller, and J. D. Smith.** 2005. Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. J Infect. Dis. **191**:1010–1013.
24. **Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, J. A. Eisen, K. Rutherford, S. L. Salzberg, A. Craig, S. Kyes, M. S. Chan, V. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen, J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser, and B. Barrell.** 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **419**:498–511.
25. **Gerlitz, B., T. Hassell, C. J. Vlahos, J. F. Parkinson, N. U. Bang, and B. W. Grinnell.** 1993. Identification of the predominant glycosaminoglycan-attachment site in soluble recombinant human thrombomodulin: potential regulation of functionality by glycosyltransferase competition for serine474. Biochem. J. **295**:131–140.
26. **Gowda, A. S., S. V. Madhunapantula, R. N. Achur, M. Valiyaveettil, B. P. Veer, and D. C. Gowda.** 2007. Structural basis for the adherence of *Plasmodium falciparum* infected erythrocytes to chondroitin 4-sulfate and design of novel photoactivable reagents for the identification of parasite adhesive proteins. J. Biol. Chem. **282**:916–928.
27. **Guyatt, H. L., and R. W. Snow.** 2004. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. Clin. Microbiol. Rev. **17**:760–769.
28. **Gysin, J., B. Pouvelle, N. Fievet, A. Scherf, and C. Lepolard.** 1999. Ex vivo desequestration of *Plasmodium falciparum*-infected erythrocytes from human placenta by chondroitin sulfate A. Infect. Immun. **67**:6596–6602.
29. **Haldar, K., and N. Mohandas.** 2007. Erythrocyte remodeling by malaria parasites. Curr. Opin. Hematol. **14**:203–209.
30. **Hiller, N. L., S. Bhattacharjee, C. van Ooij, K. Liolios, T. Harrison, C. Lopez-Estrano, and K. Haldar.** 2004. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science **306**:1934–1937.
31. **Jensen, A. T., H. D. Zornig, C. Buhmann, A. Salanti, K. A. Koram, E. M. Riley, T. G. Theander, L. Hviid, and T. Staalsoe.** 2003. Lack of gender-specific antibody recognition of products from domains of a *var* gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. Infect. Immun. **71**:4193–4196.
32. **Knuepfer, E., M. Rug, N. Klonis, L. Tilley, and A. F. Cowman.** 2005. Trafficking of the major virulence factor to the surface of transfected *P. falciparum*-infected erythrocytes. Blood **105**:4078–4087.
33. **Kraemer, S. M., S. A. Kyes, G. Aggarwal, A. L. Springer, S. O. Nelson, Z. Christodoulou, L. M. Smith, W. Wang, E. Levin, C. I. Newbold, P. J. Myler, and J. D. Smith.** 2007. Patterns of gene recombination shape var gene repertoires in *Plasmodium falciparum*: comparisons of geographically diverse isolates. BMC Genomics **8**:45.
34. **Kraemer, S. M., and J. D. Smith.** 2003. Evidence for the importance of genetic structuring to the structural and functional specialization of the *Plasmodium falciparum* var gene family. Mol. Microbiol. **50**:1527–1538.
35. **Kriek, N., L. Tilley, P. Horrocks, R. Pinches, B. C. Elford, D. J. Ferguson, K. Lingelbach, and C. I. Newbold.** 2003. Characterization of the pathway for transport of the cytoadherence-mediating protein, PfEMP1, to the host cell surface in malaria parasite-infected erythrocytes. Mol. Microbiol. **50**:1215–1227.
36. **Kyes, S., R. Pinches, and C. Newbold.** 2000. A simple RNA analysis method shows var and rif multigene family expression patterns in *Plasmodium falciparum*. Mol. Biochem. Parasitol. **105**:311–315.
37. **Lavstsen, T., A. Salanti, A. T. Jensen, D. E. Arnot, and T. G. Theander.** 2003. Sub-grouping of *Plasmodium falciparum* 3D7 var genes based on sequence analysis of coding and non-coding regions. Malar. J. **2**:27.
38. **Le Roch, K. G., J. R. Johnson, L. Florens, Y. Zhou, A. Santrosyan, M. Grainger, S. F. Yan, K. C. Williamson, A. A. Holder, D. J. Carucci, J. R. Yates III, and E. A. Winzeler.** 2004. Global analysis of transcript and protein levels across the *Plasmodium falciparum* life cycle. Genome Res. **14**:2308–2318.
39. **Llinas, M., and H. A. del Portillo.** 2005. Mining the malaria transcriptome. Trends Parasitol. **21**:350–352.

40. **Llinas, M., and J. L. DeRisi.** 2004. Pernicious plans revealed: *Plasmodium falciparum* genome wide expression analysis. Curr. Opin. Microbiol. **7**:382–387.
41. **Marsh, K., and R. J. Howard.** 1986. Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. Science **231**: 150–153.
42. **Marsh, K., L. Otoo, R. J. Hayes, D. C. Carson, and B. M. Greenwood.** 1989. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans. R. Soc. Trop. Med. Hyg. **83**:293–303.
43. **Marti, M., J. Baum, M. Rug, L. Tilley, and A. F. Cowman.** 2005. Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. J. Cell Biol. **171**:587–592.
44. **Marti, M., R. T. Good, M. Rug, E. Knuepfer, and A. F. Cowman.** 2004. Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science **306**:1930–1933.
45. **Mok, B. W., U. Ribacke, G. Winter, B. H. Yip, C. S. Tan, V. Fernandez, Q. Chen, P. Nilsson, and M. Wahlgren.** 2007. Comparative transcriptional analysis of isogenic *Plasmodium falciparum* clones of distinct antigenic and adhesive phenotypes. Mol. Biochem. Parasitol. **151**:184–192.
46. **Mutabingwa, T. K., M. C. Bolla, J. L. Li, G. J. Domingo, X. Li, M. Fried, and P. E. Duffy.** 2005. Maternal malaria and gravidity interact to modify infant susceptibility to malaria. PLoS Med. **2**:e407.
47. **Muthusamy, A., R. N. Achur, V. P. Bhavanandan, G. G. Fouda, D. W. Taylor, and D. C. Gowda.** 2004. *Plasmodium falciparum*-infected erythrocytes adhere both in the intervillous space and on the villous surface of human placenta by binding to the low-sulfated chondroitin sulfate proteoglycan receptor. Am. J. Pathol. **164**:2013–2025.
48. **Nunes, M. C., J. P. Goldring, C. Doerig, and A. Scherf.** 2007. A novel protein kinase family in *Plasmodium falciparum* is differentially transcribed and secreted to various cellular compartments of the host cell. Mol. Microbiol. **63**:391–403.
49. **Ofori, M. F., T. Staalsoe, V. Bam, M. Lundquist, K. P. David, E. N. Browne, B. D. Akanmori, and L. Hviid.** 2003. Expression of variant surface antigens by *Plasmodium falciparum* parasites in the peripheral blood of clinically immune pregnant women indicates ongoing placental infection. Infect. Immun. **71**:1584–1586.
50. **Oleinikov, A. V., E. Rossnagle, S. Francis, T. K. Mutabingwa, M. Fried, and P. E. Duffy.** 2007. Effects of sex, parity, and sequence variation on seroreactivity to candidate pregnancy malaria vaccine antigens. J. Infect. Dis. **196**: 155–164.
51. **Papakrivos, J., C. I. Newbold, and K. Lingelbach.** 2005. A potential novel mechanism for the insertion of a membrane protein revealed by a biochemical analysis of the *Plasmodium falciparum* cytoadherence molecule PfEMP-1. Mol. Microbiol. **55**:1272–1284.
52. **Ralph, S. A., E. Bischoff, D. Mattei, O. Sismeiro, M. A. Dillies, G. Guigon, J. Y. Coppee, P. H. David, and A. Scherf.** 2005. Transcriptome analysis of antigenic variation in *Plasmodium falciparum-var* silencing is not dependent on antisense RNA. Genome Biol. **6**:R93.
53. **Reeder, J. C., A. F. Cowman, K. M. Davern, J. G. Beeson, J. K. Thompson, S. J. Rogerson, and G. V. Brown.** 1999. The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. Proc. Natl. Acad. Sci. USA **96**:5198–5202.
54. **Ricke, C. H., T. Staalsoe, K. Koram, B. D. Akanmori, E. M. Riley, T. G. Theander, and L. Hviid.** 2000. Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. J. Immunol. **165**:3309–3316.
55. **Robinson, B. A., T. L. Welch, and J. D. Smith.** 2003. Widespread functional specialization of *Plasmodium falciparum* erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. Mol. Microbiol. **47**:1265–1278.
56. **Rogerson, S. J., J. G. Beeson, C. G. Mhango, F. K. Dzinjalamala, and M. E. Molyneux.** 2000. *Plasmodium falciparum* rosette formation is uncommon in isolates from pregnant women. Infect. Immun. **68**:391–393.
57. **Rogerson, S. J., R. Tembenu, C. Dobano, S. Plitt, T. E. Taylor, and M. E. Molyneux.** 1999. Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria. Am. J. Trop. Med. Hyg. **61**:467–472.
58. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132**:365–386.
59. **Salanti, A., M. Dahlback, L. Turner, M. A. Nielsen, L. Barfod, P. Magistrado, A. T. Jensen, T. Lavstsen, M. F. Ofori, K. Marsh, L. Hviid, and T. G. Theander.** 2004. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J. Exp. Med. **200**:1197–1203.
60. **Salanti, A., T. Staalsoe, T. Lavstsen, A. T. Jensen, M. P. Sowa, D. E. Arnot, L. Hviid, and T. G. Theander.** 2003. Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol. Microbiol. **49**:179–191.
61. **Sargeant, T. J., M. Marti, E. Caler, J. M. Carlton, K. Simpson, T. P. Speed, and A. F. Cowman.** 2006. Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. Genome Biol. **7**:R12.
62. **Scherf, A., B. Pouvelle, P. A. Buffet, and J. Gysin.** 2001. Molecular mechanisms of *Plasmodium falciparum* placental adhesion. Cell Microbiol. **3**:125–131.
63. **Schneider, A. G., and O. Mercereau-Puijalon.** 2005. A new Apicomplexa-specific protein kinase family: multiple members in *Plasmodium falciparum*, all with an export signature. BMC Genomics **6**:30.
64. **Smith, J. D., A. G. Craig, N. Kriek, D. Hudson-Taylor, S. Kyes, T. Fagan, R. Pinches, D. I. Baruch, C. I. Newbold, and L. H. Miller.** 2000. Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. Proc. Natl. Acad. Sci. USA **97**:1766–1771.
65. **Smith, J. D., B. Gamain, D. I. Baruch, and S. Kyes.** 2001. Decoding the language of var genes and *Plasmodium falciparum* sequestration. Trends Parasitol. **17**:538–545.
66. **Staalsoe, T., R. Megnekou, N. Fievet, C. H. Ricke, H. D. Zornig, R. Leke, D. W. Taylor, P. Deloron, and L. Hviid.** 2001. Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes that protect against placental parasitemia. J. Infect. Dis. **184**:618–626.
67. **Tuikue Ndam, N. G., A. Salanti, J. Y. Le-Hesran, G. Cottrell, N. Fievet, L. Turner, S. Sow, J. M. Dangou, T. Theander, and P. Deloron.** 2006. Dynamics of anti-VAR2CSA immunoglobulin G response in a cohort of senegalese pregnant women. J. Infect. Dis. **193**:713–720.
68. **Vincensini, L., S. Richert, T. Blisnick, A. Van Dorsselaer, E. Leize-Wagner, T. Rabilloud, and C. Braun Breton.** 2005. Proteomic analysis identifies novel proteins of the Maurer's clefts, a secretory compartment delivering *Plasmodium falciparum* proteins to the surface of its host cell. Mol. Cell. Proteomics **4**:582–593.
69. **Westfall, P. H., and S. Young.** 1993. Resampling-based multiple testing: exampes and methods for p-value adjustment. John Wiley and Sons, New York, NY.

Editor: J. F. Urban, Jr.

Plasmodium in the placenta: parasites, parity, protection, prevention and possibly preeclampsia

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SUMMARY

The epidemiology of pregnancy malaria infection and disease is complex but reflects underlying interactions between the *Plasmodium falciparum* parasite, the mother, and the foetus. Parasites sequester in the human placenta by binding to chondroitin sulfate A (CSA), a novel receptor that does not commonly support binding of other parasites. Women become resistant to *P. falciparum* malaria over successive pregnancies as they acquire antibodies against the CSA-binding placental parasite forms. Due to acquired immunity, placental malaria is briefer and less inflammatory in multigravid women than primigravid women, and these parity differences may account for the different outcomes these women and their offspring experience. Commonly recognized sequelae of malaria-like maternal anaemia and low birth weight primarily occur in first and second pregnancies. Hypertension may result from maternal-foetal conflict over the inflammatory response to placental malaria, and occurs in young, first-time mothers. Placental malaria can either increase or decrease parasitaemia risk in the offspring, depending on the mother's parity. The burden of disease due to pregnancy malaria, and the benefits of an effective vaccine, may be much greater than is currently appreciated.

Key words: *Plasmodium falciparum*, pregnancy, parity, placenta, preeclampsia.

INTRODUCTION

In areas of stable malaria transmission, residents acquire immunity by adulthood that limits infection and prevents severe disease due to *Plasmodium falciparum*. Immunity is compromised in first-time mothers, who frequently experience peripheral parasitaemia as well as dense accumulations of parasites and inflammatory cells in the placenta. Placental malaria is associated with poor outcomes, both for mothers who often develop severe anaemia and for newborns who are commonly underweight. Malaria-related low birth weight (LBW) is estimated to cause about 100 000–200 000 infant deaths each year (Guyatt and Snow, 2001; Murphy and Breman, 2001).

Susceptibility to malaria infection and disease during pregnancy has been a longstanding mystery. Many observers presumed that 'immunosuppression' during pregnancy is required to prevent rejection of the foetal allograft, and that this also increased susceptibility to malaria. However, this hypothesis did not explain the peculiar epidemiology of pregnancy malaria due to *P. falciparum*, in which resistance increases over successive pregnancies when women live in an area of stable malaria transmission. Antimalarial prophylaxis confers few benefits in multigravid women (Greenwood *et al.* 1992), indicating that immunosuppression is not increasing susceptibility during later pregnancies, and is therefore unlikely to be increasing susceptibility during first pregnancies.

To explain parity-related resistance, MacGregor speculated that local uteroplacental immune responses were required for protection, and that these might develop over successive pregnancies (McGregor, 1984). However, responses that localize to the uteroplacental unit have never been properly defined. Ultimately, the discovery that a distinct binding form of *P. falciparum* caused pregnancy malaria provided an explanation for many of the epidemiological and clinical features of this syndrome. The finding also offered new strategies for intervention.

A DISTINCT PARASITE PHENOTYPE CAUSES PLACENTAL MALARIA GLOBALLY

Among the four human malaria parasite species, only *P. falciparum* has the property of binding to endothelium and sequestering in deep vascular beds. Early observers deduced the nature and importance of parasite adhesion. Alphonse Laveran, who discovered the malaria parasite (Laveran, 1882), observed that the organ-specific syndromes of falciparum malaria (e.g. cerebral malaria) corresponded to the burden of malaria parasites sequestered in the affected tissue (Laveran, 1884). Detailed quantitative histological studies have supported this relationship (MacPherson *et al.* 1985). Marchiafava and Bignami – Italian contemporaries and rivals of Laveran – deduced that parasite-infected

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Parasitology (2007), **134**, 1877–1881.
doi:10.1017/S0031182007000170 Printed in the United Kingdom

erythrocytes (IE) bind to endothelial surfaces, leading to parasite sequestration (Marchiafava and Bignami, 1894).

IE are known to bind numerous host molecules, such as CD36, ICAM-1, and the glycosaminoglycan (GAG) chondroitin sulfate A (CSA) among several others. IE collected from infected individuals commonly bind CD36, sometimes in combination with other receptors. IE collected from the human placenta have a distinct binding phenotype – they bind to CSA but do not bind to CD36 (Fried and Duffy, 1996). The discovery of CSA-binding parasites in the placenta was doubly surprising, since adhesion had previously been discounted as the basis for placental sequestration (Bray and Sinden, 1979). IE in the placenta accumulate throughout the intervillous spaces of the placenta, most without obvious attachment to the placental lining (Fried and Duffy, 1996). After the discovery of placental CSA-binding parasites, CSA was shown to be distributed throughout the intervillous spaces of infected placentas, and to co-localize with placental IE (Muthusamy *et al.* 2004). These data support the notion that adhesion to CSA accounts for placental sequestration of parasites.

All studies of placental IE have identified adhesion to CSA as a common property of placental parasites. In addition to CSA, other placental receptors for parasite adhesion have been proposed, including hyaluronic acid (HA) and Fc receptors. Like CSA, HA is a GAG. Many preparations of HA contain CSA as a contaminant, and this CSA contaminant can support IE binding (Fried, Lauder and Duffy, 2000; Valiyaveettil *et al.* 2001). In those studies that used pure HA without CSA contamination, binding of placental IE to placental cryosections was not inhibited by soluble HA (Fried *et al.* 2000, 2006). A new study has found that placental IE do not bind to HA, and in detailed biochemical studies determined that HA was not detectable in placentas after removing umbilical cord tissue (Muthusamy *et al.* 2007). This indicates that HA is not available in the intervillous spaces of the placenta to support IE binding. Curiously, enzymatic digestion of placental cryosections with specific hyaluronidases inhibits binding of placental IE (Rasti *et al.* 2006). Further studies are required to substantiate a role for HA in placental sequestration.

Similarly, the role of Fc receptors in placental IE adhesion remains unclear. IE can adsorb immunoglobulin (Ig) G to its surface, and this prompted the hypothesis that IgG may act as a bridge for IE to bind to neonatal Fc receptor (nFcR) on the syncytiotrophoblast (Flick *et al.* 2001). However, nFcR is not expressed on the syncytiotrophoblast surface, does not bind IgG at physiological pH (7·4), and therefore is unlikely to mediate IE adhesion. Similarly, the distribution and binding properties of other IgG-binding molecules expressed by the placenta, such as annexin, placental alkaline phosphatase and other Fc receptors, suggest that they do not support binding of IgG at the syncytiotrophoblast surface (Simister and Story, 1997). Studies of freshly collected placental IE have yielded conflicting data as to whether placental IE may use IgG to bind to the syncytiotrophoblast (Fried *et al.* 2006; Rasti *et al.* 2006). More data are needed to resolve this controversy.

IMMUNITY TO PLACENTAL IE EXPLAINS PARITY-RELATED RESISTANCE

As noted above, resistance to pregnancy malaria is acquired over successive pregnancies. Among pregnant women living in areas of stable malaria transmission, first-time mothers have the highest frequency of parasitaemia and the most severe sequelae. The observation that placental IE have a distinct binding phenotype suggested a possible explanation for the parity-related pattern of susceptibility. Women may be naïve to CSA-binding parasites at the time of their first pregnancy, and then acquire antibodies over successive pregnancies that control this parasite form.

Immunoepidemiology studies have consistently supported this idea. Over successive pregnancies, women acquire antibodies that block binding of placental parasites (Fried *et al.* 1998*b*), and antibodies that react with the surface of CSA-binding parasites (Ricke *et al.* 2000). Antibodies that agglutinate placental IE may increase over successive pregnancies, although placental IE agglutinate poorly compared to other parasites (Beeson *et al.* 1999; Maubert *et al.* 1999). Cellular responses to CSA-binding parasites also increase as parity increases (Fievet *et al.* 2002).

Immunity to placental parasites or CSA-binding parasites is specifically related to improved outcomes. Anti-adhesion antibodies are related to protection from infection for the mother (Fried *et al.* 1998*b*), and are related to increased birth weight and gestational age for the newborn (Duffy and Fried, 2003). Antibody that reacts with the surface of CSA-binding parasites is related to increased haemoglobin levels and increased birth weight among women with chronic forms of placental malaria (Staalsoe *et al.* 2004). These findings have strongly supported the idea of a pregnancy malaria vaccine that would elicit immunity to placental IE surface antigens before first pregnancy in order to improve maternal and foetal outcomes.

MALARIA HAS DIFFERENT OUTCOMES IN DIFFERENT WOMEN

First-time mothers are more susceptible to malaria than other mothers. However, the course of malaria also differs in first-time mothers. Placental parasite

densities are significantly higher during first pregnancy. Inflammatory infiltrates in the placenta are frequently intense and sometimes massive during first pregnancy (Ordi *et al.* 1998), whereas placental malaria in multigravid women typically elicits little if any inflammatory infiltrate. Finally, the duration of placental malaria is longer in first-time mothers than other mothers, and the inflammatory cytokine responses in their placentas may extend beyond the resolution of parasitaemia (Fried *et al.* 1998*a*).

These different clinicopathological presentations profoundly modify the sequelae of pregnancy malaria for both the mother and her offspring. Well-known sequelae of pregnancy malaria, such as maternal anaemia and LBW offspring, are more frequent and severe during first pregnancy (Brabin and Rogerson, 2001). However, the sequelae of placental malaria may be substantially greater than previously recognized, in part because there may be insidious effects that have been masked by parity.

Preeclampsia is a deadly hypertensive disorder of pregnancy that, like falciparum malaria, is most frequent during first pregnancy. A possible relationship between placental malaria and preeclampsia has been unclear. In Senegal, placental malaria was more common in women with preeclampsia (Sartelet *et al.* 1996), while studies in Kenya failed to find a relationship between pregnancy malaria and preeclampsia (Shulman *et al.* 2001; Dorman *et al.* 2002). Data from Tanzania (Muehlenbachs *et al.* 2006) now suggest that both earlier studies may have been correct, because placental malaria causes hypertension in some mothers but not others. The risk of hypertension in infected women is strongly influenced by parity and age.

In Tanzania, young first-time mothers with placental malaria had significantly increased risk of hypertension (Muehlenbachs *et al.* 2006). Interestingly, hypertension in these women may result from a conflict between the mother and the foetus during the inflammatory response to placental infection. In older multigravid women, placental malaria lowered blood pressure, just as malaria lowers blood pressure in non-pregnant individuals. When data from all mothers were examined in aggregate, blood pressure measurements were similar between infected and uninfected women. The aggregate analysis masked the significant but opposing effects of placental malaria on blood pressure among different groups of women.

Similarly, important effects of pregnancy malaria on the offspring may be masked by an interaction with parity. In Cameroon, children born to mothers with placental malaria experienced parasitaemia more frequently than other children, although this relationship was not significant (Le Hesran *et al.* 1997). Studies in Tanzania confirm that the risk of parasitaemia is different among children born to infected women. However, the risk of parasitaemia may either increase or decrease, again depending on the parity of the mother. The frequency of parasitaemia increases among infants born to infected multigravid women, but the frequency decreases among infants born to infected first-time mothers (Mutabingwa *et al.* 2005).

Future studies will need to assess how pregnancy malaria modifies the risk of severe malaria and malaria-related death in offspring. Pregnancy malaria is related to perinatal mortality: chemoprophylaxis during first pregnancies reduces perinatal mortality by a quarter or more (Garner and Gulmezoglu, 2006), similar to the effect of insecticide-treated bednets (Gamble *et al.* 2007). However, the total impact of placental malaria on post-neonatal mortality remains unclear. Because LBW is a strong risk factor for infant mortality, LBW due to placental malaria has been estimated to cause 100 000 or more infants deaths each year. If placental malaria is also modifying the risk of severe malaria during early childhood, then the impact of placental malaria on children's health in tropical countries may be much greater than we currently appreciate.

SHORT-TERM AND LONG-TERM SOLUTIONS

The impact of pregnancy malaria on the health of mothers and children is great. In areas of high malaria transmission, nearly all pregnant women are likely to be infected sometime during pregnancy (Brabin, 1983). Rates of placental malaria at the time of delivery can be as high as 70%. Some effects of placental malaria are well-known, such as maternal anaemia and LBW infants. Other effects of placental malaria may be insidious, such as maternal hypertension or modified risk of malaria in the offspring.

Unfortunately, the tools available to prevent pregnancy malaria are narrowing. Insecticide-treated bed nets are a useful tool, but only reduce malaria infection and disease by about a quarter to a third, although the size of this benefit may vary in different populations (Gamble *et al.* 2006). Prophylaxis with chloroquine was a cheap and effective strategy until chloroquine-resistant parasites spread around the globe. Intermittent presumptive treatment during pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) replaced chloroquine as a preventative strategy in Africa. However, this strategy may soon falter with the rapid spread of SP-resistant parasites throughout the continent.

A vaccine is the best long-term strategy to prevent pregnancy malaria. Women develop resistance to pregnancy malaria over successive pregnancies, and this correlates with the acquisition of antibody against placental IE. A vaccine should reasonably target the surface proteins of placental IE to elicit protective immunity. The target(s) of these antibodies is (are) thought to be relatively conserved, because sera from multigravid women cross-react to

placental and CSA-binding IE collected from around the world (Fried *et al.* 1998*b*).

The gene *var2csa* is uniformly up-regulated in placental IE (Salanti *et al.* 2003). The VAR2CSA protein is a member of the PfEMP1 family of variant surface antigens, and it has become the primary focus of an international effort to develop a pregnancy malaria vaccine (see articles by Hviid and Salanti and by Scherf in this special issue). In addition to *var2csa*, a limited repertoire of transcripts (Francis *et al.* 2007) and IE membrane-associated proteins (Fried *et al.* 2007) are also preferentially expressed by placental parasites, and may contribute to the protective efficacy of a vaccine.

The next steps to develop a vaccine will be to identify the protein domains and variants that are targeted by functional antibodies, and to express these as recombinant immunogens that sensitize women to the surface antigens of placental IE. A pregnancy malaria vaccine could be given to women at the outset of their reproductive years, in order to elicit immune responses that would be boosted during encounters with CSA-binding parasites during pregnancy. Such a vaccine may provide immunological protection from the worst effects of pregnancy malaria, and the benefits would be greatest among first- and second-time mothers.

ACKNOWLEDGMENTS

PED is supported by grants from NIH (R01 AI52059, U19 AI065664) and the Bill & Melinda Gates Foundation (Grant 29202) to study pregnancy malaria pathogenesis and interventions.

REFERENCES

Beeson, J. G., Brown, G. V., Molyneux, M. E., Mhango, C., Dzinjalamala, F. and Rogerson, S. J. (1999). *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *Journal of Infectious Diseases* **180**, 464–472.

Brabin, B. J. (1983). An analysis of malaria in pregnancy in Africa. *Bulletin of the World Health Organization* **61**, 1005–1016.

Brabin, B. J. and Rogerson, S. J. (2001).The epidemiology and outcomes of maternal malaria. In *Malaria in Pregnancy: Deadly Parasite, Susceptible Host* (ed. Duffy, P. E. & Fried, M.), pp. 27–52. Taylor & Francis, New York.

Bray, R. S. and Sinden, R. E. (1979). The sequestration of *Plasmodium falciparum* infected erythrocytes in the placenta. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**, 716–719.

Dorman, E. K., Shulman, C. E., Kingdom, J., Bulmer, J. N., Mwendwa, J., Peshu, N. and Marsh, K. (2002). Impaired uteroplacental blood flow in pregnancies complicated by falciparum malaria. *Ultrasound in Obstetrics and Gynecology* **19**, 165–170.

Duffy, P. E. and Fried, M. (2003). Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. *Infection and Immunity* **71**, 6620–6623.

Fievet, N., Tami, G., Maubert, B., Moussa, M., Shaw, I. K., Cot, M., Holder, A. A., Chaouat, G. and Deloron, P. (2002). Cellular immune response to *Plasmodium falciparum* after pregnancy is related to previous placental infection and parity. *Malaria Journal* **1**, 16.

Flick, K., Scholander, C., Chen, Q., Fernandez, V., Pouvelle, B., Gysin, J. and Wahlgren, M. (2001). Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* **293**, 2098–2100.

Francis, S. E., Malkov, V. A., Oleinikov, A. V., Rossnagle, E., Wendler, J. P., Mutabingwa, T. K., Fried, M. and Duffy, P. E. (2007). Six genes are preferentially transcribed by the circulating and sequestered forms of *Plasmodium falciparum* parasites that infect pregnant women. *Infection and Immunity*, in press.

Fried, M., Domingo, G. J., Gowda, C. D., Mutabingwa, T. K. and Duffy, P. E. (2006). *Plasmodium falciparum*: chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta. *Experimental Parasitology* **113**, 36–42.

Fried, M. and Duffy, P. E. (1996). Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**, 1502–1504.

Fried, M., Hixson, K. K., Anderson, L., Ogata, Y., Mutabingwa, T. K. and Duffy, P. E. (2007). The distinct proteome of placental malaria parasites. *Molecular and Biochemical Parasitology* **155**, 57–65.

Fried, M., Lauder, R. M. and Duffy, P. E. (2000). *Plasmodium falciparum*: adhesion of placental isolates modulated by the sulfation characteristics of the glycosaminoglycan receptor. *Experimental Parasitology* **95**, 75–78.

Fried, M., Muga, R. O., Misore, A. O. and Duffy, P. E. (1998*a*). Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. *Journal of Immunology* **160**, 2523–2530.

Fried, M., Nosten, F., Brockman, A., Brabin, B. J. and Duffy, P. E. (1998*b*). Maternal antibodies block malaria. *Nature* **395**, 851–852.

Gamble, C., Ekwaru, J. P. and Ter Kuile, F. O. (2006). Insecticide-treated nets for preventing malaria in pregnancy. *Cochrane Database of Systematic Reviews* CD003755.

Gamble, C., Ekwaru, P. J., Garner, P. and Ter Kuile, F. O. (2007). Insecticide-treated nets for the prevention of malaria in pregnancy: a systematic review of randomised controlled trials. *PLoS Medicine* **4**, e107.

Garner, P. and Gulmezoglu, A. M. (2006). Drugs for preventing malaria in pregnant women. *Cochrane Database of Systematic Reviews* CD000169.

Greenwood, A. M., Armstrong, J. R., Byass, P., Snow, R. W. and Greenwood, B. M. (1992). Malaria chemoprophylaxis, birth weight and child survival. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 483–485.

Guyatt, H. L. and Snow, R. W. (2001). The epidemiology and burden of *Plasmodium*

falciparum-related anemia among pregnant women in sub-Saharan Africa. *American Journal of Tropical Medicine and Hygiene* **64**, 36–44.

Laveran, A. (1882). De la nature parasitaire de l'impaludisme. *Bulletins et memoires de la Societe Medicale des Hopitaux de Paris* **18**, 168–176.

Laveran, A. (1884). *Traite des Fievres Palustres*, 1st edn. Octave Doin, Paris.

Le Hesran, J. Y., Cot, M., Personne, P., Fievet, N., Dubois, B., Beyeme, M., Boudin, C. and Deloron, P. (1997). Maternal placental infection with *Plasmodium falciparum* and malaria morbidity during the first 2 years of life. *American Journal of Epidemiology* **146**, 826–831.

Macpherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S. and Warrell, D. A. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *American Journal of Pathology* **119**, 385–401.

Marchiafava, E. and Bignami, A. (1894). On summer-autumnal fevers. In *Two Monographs on Malaria and the Parasites of Malarial Fevers* (ed. Charles, T. E.), pp. 1–393. The New Sydenham Society, London.

Maubert, B., Fievet, N., Tami, G., Cot, M., Boudin, C. and Deloron, P. (1999). Development of antibodies against chondroitin sulfate A-adherent *Plasmodium falciparum* in pregnant women. *Infection and Immunity* **67**, 5367–5371.

McGregor, I. A. (1984). Epidemiology, malaria and pregnancy. *American Journal of Tropical Medicine and Hygiene* **33**, 517–525.

Muehlenbachs, A., Mutabingwa, T. K., Edmonds, S., Fried, M. and Duffy, P. E. (2006). Hypertension and maternal-fetal conflict during placental malaria. *PLoS Medicine* **3**, e446.

Murphy, S. C. and Breman, J. G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *American Journal of Tropical Medicine and Hygiene* **64**, 57–67.

Mutabingwa, T. K., Bolla, M. C., Li, J. L., Domingo, G. J., Li, X., Fried, M. and Duffy, P. E. (2005). Maternal malaria and gravidity interact to modify infant susceptibility to malaria. *PLoS Medicine* **2**, e407.

Muthusamy, A., Achur, R. N., Bhavanandan, V. P., Fouda, G. G., Taylor, D. W. and Gowda, D. C. (2004). *Plasmodium falciparum*-infected erythrocytes adhere both in the intervillous space and on the villous surface of human placenta by binding to the low-sulfated chondroitin sulfate proteoglycan receptor. *American Journal of Pathology* **164**, 2013–2025.

Muthusamy, A., Achur, R. N., Valiyaveettil, M., Botti, J. J., Taylor, D. W., Leke, R. F. and Gowda, D. C. (2007). Chondroitin sulfate proteoglycan but not hyaluronic acid is the receptor for the adherence of *Plasmodium falciparum*-infected erythrocytes in human placenta and infected red blood cell adherence up-regulates the receptor expression. *American Journal of Pathology* **170**, 1989–2000.

Ordi, J., Ismail, M. R., Ventura, P. J., Kahigwa, E., Hirt, R., Cardesa, A., Alonso, P. L. and Menendez, C. (1998). Massive chronic intervillositis of the placenta associated with malaria infection. *American Journal of Surgical Pathology* **22**, 1006–1011.

Rasti, N., Namusoke, F., Chene, A., Chen, Q., Staalsoe, T., Klinkert, M. Q., Mirembe, F., Kironde, F. and Wahlgren, M. (2006). Nonimmune immunoglobulin binding and multiple adhesion characterize *Plasmodium falciparum*-infected erythrocytes of placental origin. *Proceedings of the National Academy of Sciences, USA* **103**, 13795–13800.

Ricke, C. H., Staalsoe, T., Koram, K., Akanmori, B. D., Riley, E. M., Theander, T. G. and Hviid, L. (2000). Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *Journal of Immunology* **165**, 3309–3316.

Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A. T., Sowa, M. P., Arnot, D. E., Hviid, L. and Theander, T. G. (2003). Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Molecular Microbiology* **49**, 179–191.

Sartelet, H., Rogier, C., Milko-Sartelet, I., Angel, G. and Michel, G. (1996). Malaria associated pre-eclampsia in Senegal. *Lancet* **347**, 1121.

Shulman, C. E., Marshall, T., Dorman, E. K., Bulmer, J. N., Cutts, F., Peshu, N. and Marsh, K. (2001). Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. *Tropical Medicine and International Health* **6**, 770–778.

Simister, N. E. and Story, C. M. (1997). Human placental Fc receptors and the transmission of antibodies from mother to fetus. *Journal of Reproductive Immunology* **37**, 1–23.

Staalsoe, T., Shulman, C. E., Bulmer, J. N., Kawuondo, K., Marsh, K. and Hviid, L. (2004). Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated *Plasmodium falciparum* malaria. *Lancet* **363**, 283–289.

Valiyaveettil, M., Achur, R. N., Alkhalil, A., Ockenhouse, C. F. and Gowda, D. C. (2001). *Plasmodium falciparum* cytoadherence to human placenta: evaluation of hyaluronic acid and chondroitin 4-sulfate for binding of infected erythrocytes. *Experimental Parasitology* **99**, 57–65.

Am. J. Trop. Med. Hyg., 77(Suppl 6), 2007, pp. 270–275

New Interventions for Malaria: Mining the Human and Parasite Genomes

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Abstract. Malaria has been the greatest scourge of humankind for many millennia, and as a consequence has had more impact than any other pathogen in shaping the human genome. The sequencing of the human genome provides a new opportunity to determine the genetic traits that confer resistance to infection or disease. The identification of these traits can reveal immune responses, or host–parasite interactions, which may be useful for designing vaccines or new drugs. Similarly, the parasite genome sequence is being exploited to accelerate the development of new antimalarial interventions, for example by identifying parasite metabolic pathways that may be targeted by drugs. The malaria parasites are well known for their ability to undergo antigenic variation, and in parallel to cause a diverse array of disease syndromes, including the severe syndromes that commonly cause death. Genome-based technologies are being harnessed to relate gene and protein expression levels, or genetic variation, to the parasite forms that are targets of protective immunity. Well-conducted clinical studies are required to relate host or parasite diversity with disease. However, genomics studies of human populations raise important ethical issues, such as the disposition of data related to disease susceptibility or paternity, and the ability of communities to understand the nature of the research.

THE MANY MANIFESTATIONS OF MALARIA

Although human malaria results from infection with any of four *Plasmodium* species, research has primarily concerned itself with *P. falciparum* malaria, which is the deadliest kind. *P. falciparum* malaria takes a devastating toll on human life, with estimates of 1 to 3 million deaths annually. The deadly syndromes of malaria can take diverse forms, including severe anemia, respiratory distress, and cerebral malaria. The sequence of events that leads to severe disease and death is incompletely understood.

Understanding the mechanisms of disease will offer important opportunities for intervention, and to a large extent the story of these diseases must have been written into the genomes of the parasite and the host. In some cases, parasite factors underlie the different syndromes, such as the well-known property of parasites causing pregnancy malaria to bind the placental receptor chondroitin sulfate A (CSA).[1] Scientists have used the parasite genome to assist with identifying the parasite protein(s) that mediate parasite binding to CSA.[2]

Looked at from another perspective, the vast majority of malaria infections—500 million cases annually by some counts—do not result in death. By that measure, malaria does not seem especially dangerous because more than 99% of cases do not cause death. Therein lies another tale—one of parasite and host co-adaptation. In part, this story is written into the human genome, which has been selectively shaped by the malaria parasite to a greater extent than any other pathogen.[3] Otherwise deleterious genetic traits have been maintained at high frequencies in malaria-exposed populations because of the selective advantage that they confer against the disease.[4,5] Understanding how the human genome has been shaped by this experience can offer important insights into host strategies that prevent severe disease and death and whether these are conferred by innate resistance or acquired immunity.

THE GENOME PROMISE AND SOME QUESTIONS

The mechanisms of survival for both the parasite and its human host offer insights that can guide the development of malaria vaccines and antimalarial drugs. *P. falciparum* exhibits great diversity in both its phenotypic and genotypic characteristics on a world-wide scale.[6] Parasite diversity has been interpreted as a way for the parasite to escape the immune responses of the infecting human host.[7–9] Some properties of parasite-infected erythrocytes (IE) such as rosetting (binding to uninfected erythrocytes) or adhesion to different endothelial receptors have been associated with severe malaria, and are variably expressed by the parasite.

Conversely, the ability to resist *P. falciparum* malaria is an important adaptation of human populations living in endemic areas.[10] The sickle cell trait is the best studied innate mechanism of resistance to malaria. During the past 20 years, polymorphisms in multiple other human genes have been described that affect susceptibility to *P. falciparum* infections, including additional hemoglobin mutations,[11,12] HLA Bw53,[13] and Toll-like receptor polymorphisms.[14]

Unraveling the strands of these two tales—the tale of a lethal parasite and the tale of human survival—may now be possible with the completed sequences of human and *Plasmodium falciparum* genomes.[15,16] These major achievements have opened the door to rapid advancements in the research areas of comparative genomics, transcriptomics, and proteomics. Four years after the publication of the parasite genome, though, are we able to list new drug targets, new malaria vaccine candidates, and new diagnostic tools derived from this field of research?

Many post-genomics studies have been completed, and substantial data have been generated. What are the strategies for turning genome sequences or genomic technologies into malaria intervention or public health tools? What information can be derived from the parasite genome or the human genome, and which genome may contain the information that is

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necessary for a solution? Finally, how will these new technologies be integrated into clinical studies in tropical countries, and how can scientists in these countries access the tools and best design their research in a post-genomic world?

MINING THE PARASITE GENOME

Barring eradication of either malaria parasites or the mosquitoes that transmit them, the solution to the malaria problem requires effective drugs and vaccines. In recent times, we have been losing the race to stay ahead of the parasite. Deaths have mounted as chloroquine-resistant parasites spread around the world, and resistance to sulfadoxine-pyrimethamine developed soon after it was introduced to replace chloroquine in many areas. Meantime, the promise of a highly effective vaccine has remained elusive, despite some key advances including the partially effective RTS, S vaccine.[17]

The parasite genome may offer the means to stay ahead in the race against the parasite. Traditional approaches to drug development for malaria have yielded a limited pharmacopeia. Our best drugs have been derived from traditional herbal treatments, for example quinine and artemisinin, or from cumbersome small molecule library and synthetic analogue screens, such as mefloquine. As the few remaining drugs lose their efficacy—and there is worrying evidence that resistance to artemisinins may be emerging in some areas where it has been introduced[18]—we must find alternative strategies to identify new targets.

The malaria genome, of course, contains many potential drug targets. Genes that are essential for parasite survival are the natural starting point, and those encoding proteins involved in metabolic pathways are the traditional candidates. The genome sequence has greatly extended our understanding of the parasite's metabolic pathways, and thus revealed numerous targets for drug development. For example, the identification of genes involved in isoprenoid biosynthesis in the falciparum genome led to the identification of fosmidomycin and its derivatives as potential antimalarial drugs.[19]

Transfection technologies allow scientists to selectively "knockout" individual genes and to determine whether the parasite can survive without them. Notably, some genes are essential to parasite survival during blood stage development, whereas other genes are essential during the liver stage[20] or in the sexual stage (the stage that transmits to mosquitoes). Any of these may be a useful drug target. A blood stage target is ideal for treatment of sick persons, whereas a liver stage target may be useful for developing prophylactic agents.

THE PARASITE GENOME AND VACCINE DISCOVERY

The completion of the malaria genome sequence also allows a more deliberate approach to vaccine development. Antigens may be selected according to their role in protective immunity. Protective immunity can prevent infection or can prevent disease. Immunity that prevents infection can be achieved by inoculating humans with parasites attenuated by irradiation.[21–23] Immunity that prevents disease develops naturally when people are exposed to malaria over an extended period of time. For example, adult residents of areas with stable malaria transmission are able to limit parasitemia and do not often become sick when they are infected with *P. falciparum*. Passive transfer studies in humans have demonstrated that the IgG fraction of serum contains this protective activity.[24,25]

New technologies have been developed that capitalize on the available genome sequences. These include DNA microarrays to study gene transcription, proteomics tools to measure protein expression, and bioinformatics approaches that predict protein function and localization based on gene or protein sequences. These tools are high throughput approaches, and in some cases have overcome technical problems that previously stymied laboratory studies of malaria parasites. For these reasons, the genome-based technologies will be extremely useful to identify the malaria antigens that may be involved in models of protective immunity.

Surface proteins are a logical target for vaccines, because they often carry out key functions such as adhesion or invasion, and they are accessible to antibodies. Proteomics tools survey the full repertoire of surface proteins expressed by a pathogen, from which the optimal antigens for a vaccine can be rationally selected. For example, proteomics studies identified novel surface proteins of *Streptococcus agalactica* and *Helicobacter pylori*.[26,27]

In the case of *P. falciparum*, mass spectrometry (MS) has been used to characterize proteins expressed on the surface of the infected erythrocytes (IE) in fresh samples collected from Tanzanian donors.[28] Parasites form electron-dense structures called "knobs" on the IE surface that mediate adhesion to the vascular endothelium. In the study from Tanzania, knob proteins were enriched by differential detergent extraction followed by various fractionation methods prior to liquid chromatography (LC)-MS/MS. This approach identified peptides from hundreds of proteins. Among these, 75% contained a predicted transmembrane (TM) domain, and 70% had no known function. This approach identified variant surface antigens as well as several conserved proteins with predicted TM domains or signal peptides, which were associated with the CSA-binding or placental parasites.

DNA microarrays that measure gene transcription are a useful adjunct to proteomics studies. Although gene transcription levels do not necessarily correspond to protein levels, microarrays (which measure thousands of genes) have greater sensitivity than MS technologies (which measure hundreds of proteins). DNA microarrays have been applied to laboratory isolates to define the entire transcriptome of *P. falciparum* during intraerythrocytic and mosquito stages.[27,29] One approach to malaria antigen discovery entails identifying the genes that are upregulated in virulent or disease-causing parasite forms. For example, upregulated genes identified by microarray analysis of *Neisseria meningitidis* serogroup B were shown to encode proteins that protect against meningococcus in a mouse model.[30,31]

For malaria, vaccine developers will be interested in genes that are upregulated in the parasite stages or forms that are the target of protective immunity. The genes expressed by LS parasites encode proteins that are targets of protective immunity and could be used as subunit vaccines. Alternatively, these genes may be essential to liver stage development and therefore could be knocked out to produce genetically attenuated parasites that induce protective immunity but cannot cause blood stage infection and disease.[20]

Among blood-stage parasites, IEs that bind to the placental receptor CSA have been shown to be commonly involved in

malaria of pregnant women. For a vaccine against pregnancy malaria, scientists are using microarrays to identify the subset of genes that are preferentially expressed by CSA-binding or placental parasites.[32] The parasite forms that cause the severe malaria syndromes of children remain unknown. However, there is intense interest to identify these parasite forms because the antigens that these parasite forms express may be useful to develop vaccines that prevent disease and death due to malaria in young African children.

HUMAN GENOMIC ANALYSIS OF MALARIA RESISTANCE

Given the importance of environment and the likely importance of parasite strain variation in determining the risk of malaria, it may seem at first unlikely that human genetic variation would play a major role. However, the strongest known determinant of severe malaria due to *P. falciparum* is a human genetic factor, sickle cell trait, which reduces risk by approximately 10-fold.[13,33] Host genetic factors are estimated to account for 25% of variation in risk of hospital admission for malaria among Kenyan children, whereas household accounts for only 14% of the variation.[34] Risk of malaria infection appears to have a similar contribution from host genetics (24%) and household (29%). Importantly, sickle cell trait accounted for only 2% of the total variation, implying that many other host genetic determinants remain to be discovered.

Although human genetics is now being used as an investigative tool in many diseases, malaria may prove to be the most productive application of this approach because it is the strongest known force for evolutionary selection in the recent history of the human genome. In addition to the HbS allele, which encodes sickle hemoglobin, a remarkable range of genetic variants of erythrocyte proteins have risen to high frequency in human populations due to their protective effect against *P. falciparum* malaria. They include variants of globin structure (HbS, HbC, HbE), globin regulation (thalassemias), band III protein (ovalocytosis), and glucose-6-phosphate dehydrogenase (reviewed in ref 3). Malaria resistance alleles have served as an important paradigm in the development of statistical methods to uncover signatures of recent positive selection in the human genome.[35–38]

An elegant demonstration of how genetic discoveries can assist vaccine development is provided by Duffy blood group antigen. Some 30 years ago it was discovered that the reason for the near absence of *P. vivax* from sub-Saharan Africa is due to the fact that most of the population have a genetic variant that prevents the Duffy blood group antigen being expressed on erythrocytes.[39] This genetic discovery led to the molecular discovery of the protein that the parasite uses to bind to Duffy antigen,[40] which is essential for erythrocyte invasion by *P. vivax* and represents an obvious target for a *P. vivax* vaccine.[41]

Knowledge of the human genome sequence has revolutionized the investigation of genetic diversity within human populations. Of the 3 billion base pairs in the human genome, in the order of 10 million (i.e., approximately 1 in 300) base pairs are estimated to have polymorphisms present at more than 1% frequency in human populations.[38] Armed with this knowledge, the medical research community is investing huge efforts in large-scale epidemiologic studies to uncover the genetic basis of common human diseases. The long-term goal is not just to make genetic discoveries, but also to use these discoveries to dissect the complex web of molecular pathways that underlie the evolution of disease, as well as the molecular mechanisms that protect against disease, and thereby to accelerate the process of vaccine and drug discovery.

How can we most efficiently and reliably discover novel malaria resistance alleles? The most popular approach is candidate gene analysis, in which the investigators begin by selecting genes or gene regions of particular interest, and then type polymorphisms in those genes for association with severe malaria. The advent of PCR-based genotyping methods has led to dozens of candidate gene studies of malaria over the past 15 years or so, many of which have identified putative disease associations (reviewed in ref 3).

However, there are some problems in interpreting this mass of candidate gene data. One problem is that in very few studies have the candidate genes been tested for more than a handful of known polymorphisms, so a negative result may simply mean that important polymorphisms were omitted from the analysis. This problem will eventually be solved as more polymorphisms are discovered, and as new technologies make it possible for investigators to type many polymorphisms at affordable cost. A more serious problem is that many candidate gene studies are underpowered and have not been replicated in independent studies, leading to a high rate of both false negative and false positive results. Population stratification is an important cause of false positives in case-control studies. It arises when the cases and controls are sampled from sub-populations of different ancestry, so that the apparent disease association simply reflects the different genetic makeup of the sub-populations rather than genetic effects on the disease itself. It is thus imperative to match cases and controls by ethnic group, but even with careful matching it may be difficult to avoid subtle stratification effects in ethnically heterogeneous populations.[42] Family-based association studies, in which the genotypes of an affected child are compared with those of its parents, provide a way of excluding these population artifacts and represent an important complementary approach to standard case-control analysis.[33]

The cutting edge of genomic epidemiology goes beyond candidate genes to the whole human genome, which harbors thousands of genes whose function is unknown, some of which may play a critical role in host–parasite interactions. Discovery of novel genes that influence the evolution of disease and of protective immunity could provide vital clues for vaccine development, as illustrated by the example of Duffy antigen given previously. Screening the whole human genome for malaria resistance alleles by genetic association analysis would not have been feasible a few years ago, but 3 recent advances now make this possible. First, the International HapMap Project has surveyed almost 4 million single nucleotide polymorphisms (SNPs) across the genome, and by describing the common combinations of alleles found in individuals of African, Asian, and European origin, provides a rational basis for the identification of SNPs that are informative about common forms of human variation in different populations.[38] Second, new high-throughput genotyping technologies allow an individual to be genotyped for over half a million SNPs in a single assay. Finally, the establishment of large, multicenter collaborations such as the Malaria Ge-

nomic Epidemiology Network (www.malariagen.net) makes it feasible to achieve the sample sizes required to carry out genome-wide association studies that are adequately powered to correct for the vast number of multiple observations.[43] According to current thinking this needs at least 2,000 cases and 2,000 controls, and the formation of large-scale partnerships allows this to be increased by at least 4-fold to detect genetic effects of modest magnitude.

GENOME SCIENCE IN THE FIELD

The broad intention of human and malaria parasite genetic studies is to explain observed differences in malaria disease incidence, prevalence and severity, and to apply accrued knowledge to disease control. These studies involve human subjects by collecting blood specimens and clinical information, and therefore they should meet all Good Clinical Practice principles. Some genetic studies are designed to be nested in ongoing trials or observational studies, which have separately received ethical approval.

However, many of these genomic studies are commonly introduced into the research plan at later stages of the main study. In general, genome-based studies must be performed in well-equipped laboratories, meaning that these are mostly conducted outside the endemic countries in Africa or elsewhere. In these cases, the collection and management of specimens and clinical information raise several ethical issues. How does the study team obtain valid written informed consent for samples meant for future studies? What measures are in place to ensure that samples transferred to another laboratory or country are strictly used for intended and stated investigations?

Practical issues also must be considered. The team must be prepared to manage the cross-border transportation of genetic materials. Thought must be given as well to the nature of the genetic information being collected, and the manner in which this information may be returned to the study participants. Study teams should be particularly aware of unearthing sensitive human genetic findings, such as information on genetic traits associated with disease susceptibility, or family information that may determine paternity of children. Although these ethical issues have no clear cut answers, they should be adequately addressed by the study team in collaboration with local Institutional Review Boards (IRBs) and Ethics Review Committees (ERCs).

Apart from scientists, lay populations are also aware that disease severity varies within communities exposed to similar intensity of transmission and presumably malaria parasites of similar virulence. The awareness of the lay population is a good thing because this will facilitate their understanding of the basis for genomics studies as well as obtaining informed consent for the studies.

Inter-individual variations in disease manifestations under similar epidemiologic settings may be explained in part by variations in the parasite and in part by genetic determinants in the host. Although current knowledge indicates that some polymorphisms within the human genome such as hemoglobinopathies and certain thalassemias confer protection to malaria, the true mechanisms of protection remains unclear. Understanding these mechanisms is wholly dependent on good clinical studies, partnered with strong basic research in genomics, pathogenesis, and immunology.

CONCLUSIONS

The genome sequences of the malaria parasite *P. falciparum* and its human host have created new opportunities to solve the malaria problem. Although the availability of genome sequences and genome-based technologies have already greatly expanded our understanding of both the parasite and the host response to it, the new genomic era has yet to deliver on its promise of effective new drugs and vaccines. The solution to the malaria problem lies in an understanding of both parasite and human genomes, and the insights these offer into the myriad interactions between the parasite and the host. In many cases, the full realization of the genomic promise will require the application of these technologies to populations affected by malaria, meaning that studies designed to meet the highest clinical and ethical standards must be conducted in resource-poor communities. Laboratory and clinical researchers around the globe are ready to embrace the opportunities created by the genome sequences. Most people expect that a wealth of information emanating from these studies will profoundly enhance our understanding of the malaria problem. What remains less clear is where in the parasite or human genomes we may find solutions to the malaria problem, hidden right in front of us. Finding those solutions is the very reason for research.

Acknowledgments: DPK and MD thank their colleagues in the Malaria Genomic Epidemiology Network for collaborations. PED thanks M. Fried for ideas and discussions.

Financial support: FN is a member of the Malaria Immunology and Pathogenesis Network (MIMPAC). DPK receives support from the Medical Research Council, Wellcome Trust, Bill and Melinda Gates Foundation and Grand Challenges in Global Health. MD receives support from the International Atomic Energy Authority and the Bill & Melinda Gates Foundation. PED receives support from Bill & Melinda Gates Foundation (Grant 29202), Grand Challenges in Global Health/ Foundation for NIH (Grant 1364), US Department of Defense (Award W81XWH-05-2-0014), the US National Institutes of Health (R01AI52059 and U19AI065664), and the Fogarty International Center/NIH (D43 TW05509-04).

Received August 21, 2006. Accepted for publication April 14, 2007.

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REFERENCES

1. Fried M, Duffy PE, 1996. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science 272:* 1502–1504.
2. Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, Hviid L, Theander TG, 2003. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol 49:* 179–191.
3. Kwiatkowski DP, 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet 77:* 171–192.

4. Haldane J, 1948. The rate of mutation of human genes. *Hereditas 35:* 267–273.
5. Nagel RL, Roth EF Jr, 1989. Malaria and red cell genetic defects. *Blood 74:* 1213–1221.
6. Kemp DJ, Cowman AF, Walliker D, 1990. Genetic diversity in Plasmodium falciparum. *Adv Parasitol 29:* 75–149.
7. Bull PC, Pain A, Ndungu FM, Kinyanjui SM, Roberts DJ, Newbold CI, Marsh K, 2005. *Plasmodium falciparum* antigenic variation: relationships between in vivo selection, acquired antibody response, and disease severity. *J Infect Dis 192:* 1119–1126.
8. Ntoumi F, Flori L, Mayengue PI, Matondo Maya DW, Issifou S, Deloron P, Lell B, Kremsner PG, Rihet P, 2005. Influence of carriage of hemoglobin AS and the Fc gamma receptor IIa-R131 allele on levels of immunoglobulin G2 antibodies to *Plasmodium falciparum* merozoite antigens in Gabonese children. *J Infect Dis 192:* 1975–1980.
9. Takala SL, Escalante AA, Branch OH, Kariuki S, Biswas S, Chaiyaroj SC, Lal AA, 2006. Genetic diversity in the Block 2 region of the merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum:* additional complexity and selection and convergence in fragment size polymorphism. *Infect Genet Evol 6:* 417-424.
10. Miller LH, Good MF, Milon G, 1994. Malaria pathogenesis. *Science 264:* 1878–1883.
11. Yuthavong Y, Wilairat P, 1993. Protection against malaria by thalassaemia and haemoglobin variants. *Parasitol Today 9:* 241–245.
12. Weatherall DJ, Miller LH, Baruch DI, Marsh K, Doumbo OK, Casals-Pascual C, Roberts DJ, 2002. Malaria and the red cell. *Hematology (Am Soc Hematol Educ Program):* 35–57.
13. Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, Greenwood BM, 1991. Common west African HLA antigens are associated with protection from severe malaria. *Nature 352:* 595–600.
14. Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, Otchwemah RN, Dietz E, Ehrhardt S, Schroder NW, Bienzle U, Schumann RR, 2006. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. *Proc Natl Acad Sci USA 103:* 177–182.
15. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B, 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature 419:* 498–511.
16. Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, Sauerwein RW, Eling WM, Hall N, Waters AP, Stunnenberg HG, Mann M, 2002. Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry. *Nature 419:* 537–542.
17. Heppner DG Jr, Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, Stewart VA, Dubois P, Lanar DE, Krzych U, Moris P, Angov E, Cummings JF, Leach A, Hall BT, Dutta S, Schwenk R, Hillier C, Barbosa A, Ware LA, Nair L, Darko CA, Withers MR, Ogutu B, Polhemus ME, Fukuda M, Pichyangkul S, Gettyacamin M, Diggs C, Soisson L, Milman J, Dubois MC, Garcon N, Tucker K, Wittes J, Plowe CV, Thera MA, Duombo OK, Pau MG, Goudsmit J, Ballou WR, Cohen J, 2005. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine 23:* 2243–2250.
18. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O, 2005. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. *Lancet 366:* 1960–1963.
19. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E, 1999. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science 285:* 1573–1576.
20. Mueller AK, Labaied M, Kappe SH, Matuschewski K, 2005. Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. *Nature 433:* 164–167.
21. Clyde DF, Most H, McCarthy VC, Vanderberg JP, 1973. Immunization of man against sporozite-induced falciparum malaria. *Am J Med Sci 266:* 169–177.
22. Herrington D, Davis J, Nardin E, Beier M, Cortese J, Eddy H, Losonsky G, Hollingdale M, Sztein M, Levine M, Nussenzweig RS, Clyde D, Edelman R, 1991. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. *Am J Trop Med Hyg 45:* 539–547.
23. Nussenzweig RS, Vanderberg J, Most H, Orton C, 1967. Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. *Nature 216:* 160–162.
24. Cohen S, McGregor IA, Carrington S, 1961. Gamma-globulin and acquired immunity to human malaria. *Nature 192:* 733–737.
25. McGregor IA, Carrington SP, 1963. Treatment of East African *P. falciparum* malaria with west African human γ-globulin. *Trans R Soc Trop Med Hyg 57:* 170–175.
26. Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, Younes ZN, Dobson RJ, Everest P, Reason AJ, Redfern JM, Greer FM, Paxton T, Panico M, Morris HR, Feldman RG, Santangelo JD, 2002. Identification of major outer surface proteins of Streptococcus agalactiae. *Infect Immun 70:* 1254–1259.
27. Larsson T, Bergstrom J, Nilsson C, Karlsson KA, 2000. Use of an affinity proteomics approach for the identification of low-abundant bacterial adhesins as applied on the Lewis(b)-binding adhesin of Helicobacter pylori. *FEBS Lett 469:* 155–158.
28. Fried M, Wendler JP, Mutabingwa TK, Duffy PE, 2004. Mass spectrometric analysis of *Plasmodium falciparum* erythrocyte membrane protein-1 variants expressed by placental malaria parasites. *Proteomics 4:* 1086–1093.
29. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, De La Vega P, Holder AA, Batalov S, Carucci DJ, Winzeler EA, 2003. Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science 301:* 1503–1508.
30. Grifantini R, Bartolini E, Muzzi A, Draghi M, Frigimelica E, Berger J, Ratti G, Petracca R, Galli G, Agnusdei M, Giuliani MM, Santini L, Brunelli B, Tettelin H, Rappuoli R, Randazzo F, Grandi G, 2002. Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays. *Nat Biotechnol 20:* 914–921.
31. Grifantini R, Bartolini E, Muzzi A, Draghi M, Frigimelica E, Berger J, Randazzo F, Grandi G, 2002. Gene expression profile in Neisseria meningitidis and Neisseria lactamica upon host-cell contact: from basic research to vaccine development. *Ann NY Acad Sci 975:* 202–216.
32. Francis SE, Malkov VA, Oleinikor AV, Rossnagle E, Wendler JP, Mutabingwa TK, Fried M, Duffy PE, 2007. Six genes are preferentially transcribed by the circulating and sequested forms of *Plasmodium falciparum* parasites that infect pregnant women. *Infect Immun 75:* 4838–4850.
33. Ackerman H, Usen S, Jallow M, Sisay-Joof F, Pinder M, Kwiatkowski DP, 2005. A comparison of case-control and family-based association methods: the example of sickle-cell and malaria. *Ann Hum Genet 69:* 559–565.
34. Mackinnon MJ, Mwangi TW, Snow RW, Marsh K, Williams TN, 2005. Heritability of malaria in Africa. *PLoS Med 2:* e340.
35. Tishkoff SA, Varkonyi R, Cahinhinan N, Abbes S, Argyropoulos G, Destro-Bisol G, Drousiotou A, Dangerfield B, Lefranc G, Loiselet J, Piro A, Stoneking M, Tagarelli A, Tagarelli G, Touma EH, Williams SM, Clark AG, 2001. Haplotype diversity and linkage disequilibrium at human G6PD: recent origin of alleles that confer malarial resistance. *Science 293:* 455–462.
36. Sabeti PC, Reich DE, Higgins JM, Levine HZ, Richter DJ, Schaffner SF, Gabriel SB, Platko JV, Patterson NJ, McDonald GJ, Ackerman HC, Campbell SJ, Altshuler D, Cooper R, Kwiatkowski D, Ward R, Lander ES, 2002. Detecting recent posi-

tive selection in the human genome from haplotype structure. *Nature 419:* 832–837.
37. Ohashi J, Naka I, Patarapotikul J, Hananantachai H, Brittenham G, Looareesuwan S, Clark AG, Tokunaga K, 2004. Extended linkage disequilibrium surrounding the hemoglobin E variant due to malarial selection. *Am J Hum Genet 74:* 1198–1208.
38. Altshuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, Donnelly P, 2005. A haplotype map of the human genome. *Nature 437:* 1299–1320.
39. Miller LH, Mason SJ, Clyde DF, McGinniss MH, 1976. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood- group genotype, FyFy. *N Engl J Med 295:* 302–304.
40. Chitnis CE, Miller LH, 1994. Identification of the erythrocyte binding domains of *Plasmodium vivax* and Plasmodium knowlesi proteins involved in erythrocyte invasion. *J Exp Med 180:* 497–506.
41. Yazdani SS, Shakri AR, Pattnaik P, Rizvi MM, Chitnis CE, 2006. Improvement in yield and purity of a recombinant malaria vaccine candidate based on the receptor-binding domain of *Plasmodium vivax* Duffy binding protein by codon optimization. *Biotechnol Lett. 28:* 1109-1114.
42. Marchini J, Cardon LR, Phillips MS, Donnelly P, 2004. The effects of human population structure on large genetic association studies. *Nat Genet 36:* 512–517.
43. Chokshi DA, Parker M, Kwiatkowski DP, 2006. Data sharing and intellectual property in a genomic epidemiology network: policies for large-scale research collaboration. *Bull World Health Organ 84:* 382–387.

Malaria Journal

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Malaria Journal 2008, **7**:26 doi:10.1186/1475-2875-7-26

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ISSN 1475-2875

Article type Research

Submission date 4 October 2007

Acceptance date 29 January 2008

Publication date 29 January 2008

Article URL http://www.malariajournal.com/content/7/1/26

Maternal peripheral blood level of IL-10 as a marker for inflammatory placental malaria

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Abstract

Background

Placental malaria (PM) is an important cause of maternal and foetal mortality in tropical areas, and severe sequelae and mortality are related to inflammation in the placenta. Diagnosis is difficult because PM is often asymptomatic, peripheral blood smear examination detects parasitemia as few as half of PM cases, and no peripheral markers have been validated for placental inflammation.

Methods

In a cohort of Tanzanian parturients, PM was determined by placental blood smears and placental inflammation was assessed by histology and TNF mRNA levels. Maternal peripheral blood levels of several immune mediators previously implicated in PM pathogenesis, as well as ferritin and leptin were measured. The relationship between the levels of these soluble factors to PM and placental inflammation was examined.

Results

Peripheral levels of TNF, TNF-RI, TNF-RII, IL-1, IL-10, and ferritin were elevated during PM, whereas levels of IFN-γ, IL-4, IL-5 and IL-6 were unchanged and levels of leptin were decreased. In receiver operating characteristic curve analysis, IL-10 had the greatest area under the curve, and would provide a sensitivity of 60% with a false positive rate of 10%. At a cut off level of 15 pg/mL, IL-10 would detect PM with a sensitivity of 79.5% and a specificity of 84.3%. IL-10 levels correlated with placental inflammatory cells and placental TNF mRNA levels in first time mothers.

Conclusions

These data suggest that IL-10 may have utility as a biomarker for inflammatory PM in research studies, but that additional biomarkers may be required to improve clinical diagnosis and management of malaria during pregnancy.

Background

Placental malaria (PM) due to *Plasmodium falciparum* is a major cause of mortality for mothers and their offspring, and is most frequent and severe during first pregnancies [1]. PM is caused by parasite-infected erythrocytes that bind to chondroitin sulfate A (CSA) and sequester in the placenta [2]. In histologic studies, PM can appear as an acute condition with little to no inflammation, or as a chronic disorder with sometimes heavy inflammation and deposition of parasite haemozoin (also called pigment) [3]. Chronic inflammatory PM has been most closely related to poor maternal and foetal outcomes in earlier studies [4]. In areas of stable malaria transmission, first time mothers often develop chronic PM, with inflammatory infiltrates and elevated Type 1 cytokines in the placenta [4, 5].

Antenatal diagnosis of PM by Giemsa-stained blood smears fails to identify a substantial proportion of PM cases [6], possibly as many as half [1] and no tools exist that can predict poor pregnancy outcomes. PCR-based detection of *P. falciparum* DNA in peripheral blood is frequently positive when peripheral blood smear is negative. However, PCR can detect dead parasites, free parasite DNA, or DNA in phagocytic cells, and PCR-detection is not associated with pregnancy outcomes [6]. Antigen capture tests show promise, but they yield information only on parasitaemia and not inflammation [7]. A recent study from Kenya reported an association between plasma urokinase receptor levels measured at delivery and low birth weight in maternal malaria [8], suggesting that host biomarkers may be useful for discriminating women likely to experience poor

outcomes from other women. Peripheral biomarkers of placental inflammation may be of particular value, since this condition is related to poor outcomes. In the present study peripheral blood levels of several immune mediators and other proteins in a cohort of Tanzanian women was examined at the time of delivery, and their associations with PM and placental inflammation was determined.

Methods

Clinical procedures

Placental samples, peripheral blood and clinical information were provided by Tanzanian women aged 18 to 45 years delivering at the Muheza Designated District Hospital, Muheza, Tanga region, in an area of intense malaria transmission. These women were participating in a birth cohort study known locally as the Mother-Offspring Malaria Studies (MOMS) Project. Women signed an informed consent form before joining the study, and women with known HIV or HIV-related sequelae in their offspring were excluded. Routine microbiological testing for other infectious diseases was not performed at the study site. Clinical information was collected by project nurses and assistant medical officers on standardized forms. Study procedures involving human subjects were approved by the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health, and ethical clearance was obtained from the Institutional Review Boards of Seattle Biomedical Research Institute and the National Institute for Medical Research in Tanzania.

Peripheral blood was collected in citrate phosphate dextrose around the time of delivery, and plasma was separated and frozen at -80°C. The placenta was collected at delivery, and a full thickness biopsy from the middle third of the placental disc was taken. Tissue was fresh frozen in liquid nitrogen and stored at -80°C. Placental blood samples were obtained by manual compression of the placental tissue in a grinder. Placental parasitaemia was defined as the identification of any parasites in a placental blood slide by microscopy. Thick and thin smears were prepared; thin smears were fixed with

methanol. Blood slides were stained for 10 minutes in 10% Giemsa, washed in tap water, air-dried, then examined using light microscopy at 1000 x magnification. Ten thousand red cells were examined in the thin smear before concluding that a placental blood slide was negative.

Laboratory procedures

Plasma levels of cytokines, cytokine receptors, ferritin and leptin were analyzed using a multiplexed, bead-based platform (BioPlex®, BioRad, Irvine, CA) and custom-made assay kits as previously described [9]. Detection limits for these assays were as follows: TNF 0.10 pg/ml, TNF receptor (R) I 1.58 pg/ml, TNF-RII 0.21 pg/ml, IFN-γ 0.04 pg/ml, IL-1 0.01 pg/ml, IL-4 0.30 pg/ml, IL-5 0.02 pg/ml, IL-6 0.45 pg/ml, IL-10 0.02 pg/ml, ferritin 0.07 ng/ml, and leptin 1.28 pg/ml. Levels of soluble factors were adjusted to account for dilution in anticoagulant at the time of sample collection. For each plasma sample, all analytes were assayed in a single day, thus eliminating freeze/thaw cycles.

For histologic analysis, PM-positive tissue was selected and 5 μm cryosections of placental tissue were fixed in methanol and stained with Giemsa. Sections were assessed by examining greater than ninety 600 x fields per section. Immune infiltrates within the intervillous spaces were qualitatively scored as (-) for none or very few inflammatory cells present, (+) for inflammatory cells present. Histological analysis was performed by a single observer (A.M.).

Quantitative PCR was performed as described elsewhere [10]. Briefly total RNA was extracted from frozen cryosections using RNeasy minikits (Qiagen) and cDNA was synthesized using Superscript III enzyme (Invitrogen) and anchored oligodT20 primers. Real-time PCR was performed in duplicate using SYBR green master mix and an ABI Prism 7000 or 7500 (Applied Biosystems). Threshold cycles (CT) were calculated and normalized to CT of KRT7 (a gene expressed by trophoblasts and not by inflammatory cells). Data are presented as fold-difference from control gene, calculated by $2^{(\text{control CT-gene CT})}$. The oligonucleotide primers used for PCR reactions included: TNF Forward CACGCTCTTCTGCCTGCT; TNF-α Reverse CAGCTTGAGGGTTTGCTACA; KRT7 forward: GGCTGAGATCGACAACATCA; KRT7 reverse: CTTGGCACGAGCATCCTT.

Statistical analysis

Student's t-test was used for the analysis of maternal age and birth weight within primigravid (first pregnancy) and multigravid (second and later pregnancy) groups. Mann-Whitney test was used to examine cytokine levels. Linear regression coefficients were calculated using simple regression analysis. Receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses were performed with IL-10 and other soluble factors levels as continuous variables using JROCFIT and JLABROC4 algorithms that are available online at the URL [11]. Sensitivities and specificities of elevated IL-10 to detect PM were calculated at specific cutoff levels of 10 pg/ml, 15 pg/ml or 35 pg/ml. Other analyses were performed using Statview 5.0.1 (SAS Institute, Cary, North Carolina, United States).

Results

Peripheral plasma samples used for these studies were provided by 660 women delivering singleton live-born babies in Muheza, Tanzania. Clinical data are shown in Table 1. PM+ multigravid women were younger than PM- multigravid women, and birthweight was significantly lower in PM+ deliveries compared to PM- deliveries in both gravidity groups.

Peripheral levels of cytokines, leptin and ferritin vary during PM

Comparison of concentrations of cytokines and other soluble factors in maternal peripheral blood stratified for PM and parity is shown in Table 2. PM significantly increased peripheral levels of TNF, TNF-RII, IL-10 and ferritin in women of both parities. Peripheral levels of TNF-RI and IL-1 significantly increased while levels of leptin significantly decreased in primigravid but not multigravid women during PM. The levels of other soluble factors were similar between PM- and PM+ women.

Peripheral IL-10 levels are markers of PM and placental inflammation

The soluble factors that were significantly elevated in peripheral blood during PM were analyzed by ROC curve analysis to determine their utility as biomarkers to detect PM (Table 3). IL-10 had the greatest area under the curve (AUC) at 0.83 in first time mothers and 0.82 for all mothers, indicating the highest sensitivity and specificity. The ROC curve for IL-10 in first time mothers is shown in Figure 1. Using an IL-10 cutoff for

a false positive rate of 10% would yield a sensitivity of 60%, whereas a cut off for sensitivity of 90% would yield a false positive rate of 50%. Ferritin and TNF-RII had AUC values greater than 0.75 in first time mothers. Derived values, resulting from the combination by summation or addition of IL-10 with either ferritin or TNF-RII provided no improvement in sensitivity and specificity (data not shown).

The ability of IL-10 elevations above various threshold values to discriminate infected from uninfected women was examined (Table 4). An IL-10 cutoff level of 15 pg/mL yielded values above 75% for both parameters. Peripheral IL-10 levels were specifically elevated in first time mothers who had placental inflammation by histology (Figure 2). Further, peripheral IL-10 levels correlated significantly with placental TNF mRNA (Figure 3).

Discussion

Peripheral blood smear analysis has low sensitivity to detect PM. PCR based and antigen capture tests for the diagnosis of PM have increased sensitivity but cannot detect inflammation, which is related to poor pregnancy outcomes. This study suggests that peripheral IL-10 levels may be a useful tool to identify women with inflammatory PM and therefore those likely to have poor pregnancy outcomes. Using a cut-off level of 15 pg/mL, IL-10 levels would detect PM with a sensitivity of 79.5% and specificity of 84.3%. IL-10 may have utility in longitudinal studies, examining the burden of malaria over gestation, when the placenta is not available for microscopic analysis. Future studies should measure IL-10 levels throughout gestation to assess relationships to antenatal parasitemia and to pregnancy outcomes.

IL-10 is a key cytokine both in protection and immunopathology during malaria. High levels of IL-10 observed during malarial episodes may be beneficial by reducing the inflammatory response, but may be detrimental by decreasing antiparasitic cellular immune responses. IL-10 is an anti-inflammatory cytokine that acts in part by blocking monocyte/macrophage production of inflammatory cytokines such as IL-6, TNF, and IL-1 [12]. Animal studies have suggested that IL-10 may play a regulatory role during parasitic infection that modulates susceptibility. In particular, IL-10 inhibits the microbicidal activity of IFN-γ-treated macrophages against intracellular parasites such as *Toxoplasma gondii* [13], *Trypanosoma cruzi* [14] and *Leishmania major* [15] and the

killing of extracellular *Schistosoma mansoni* schistosomulas [16]. These effects may result from decreased production of the toxic nitrogen oxide metabolites[17].

The blood stages of *P. falciparum* are also cleared by phagocytosis and killed by oxidative products of nitric oxide released by macrophages [18]. IL-10 has been previously observed to be elevated during malarial episodes in non-pregnant [19], [20] and pregnant individuals [21]. Both increased and decreased levels of IL-10 have been associated with poor malaria outcomes. Low levels of IL-10 or low IL-10 to TNF ratios were associated with severe malarial anemia in African children [22], [23] while high IL -10 levels were associated with reduced ability to eliminate malaria parasitaemia in Tanzanian children [24] .

PM results from the accumulation of parasites that bind to CSA in the intervillous spaces of the placenta [2, 25]. In response to the sequestered mass of parasites, inflammatory cells infiltrate the intervillous spaces This inflammatory infiltrate can be massive, and prominently features monocytes/ macrophages. In vitro data suggests these cells are the principal source of IL-10 [21]. In Kenyan children, high levels of peripheral blood IL-10 were positively correlated with binding of infected red blood cells to CD36 [26], but the relevance of this observation to malaria pathogenesis is unknown, and we find that levels of IL-10 also increase when CSA-binding parasites are the major parasite form causing infection. Placental levels of TNF increase during PM [5], [21], [27] and TNF gene expression is specifically related to placental inflammation [10]. Increased placental blood levels of TNF are related to poor outcomes for both the mother and her newborn

[5], [27]. In the present study, placental TNF mRNA positively correlated to peripheral blood IL-10 levels in first-time mothers, strengthening the association between peripheral IL-10 levels and placental inflammation.

The present data indicate that peripheral ferritin levels are also elevated during PM. Ferritin is a positive acute phase protein and is known to increase during infection and injury. In non-pregnant individuals, ferritin levels increase during both asymptomatic and symptomatic malaria, and the highest levels have been recorded in individuals with severe disease [28]. Serum ferritin may also increase in the presence of subclinical infection [29]. During the acute phase response, inflammatory cytokines such as IL-1ß increase the synthesis of both heavy and light subunits of ferritin [30]. In this Tanzanian cohort, PM was associated with elevated levels of IL-1 and TNF in maternal peripheral blood, particularly among first time mothers who are most likely to experience placental inflammation. Ferritin is widely used for determining iron deficiency anemia in industrialized countries, and therefore has the advantage of existing diagnostic platforms. For this reason, ferritin should also be evaluated in prospective studies as a cost-effective antenatal assay for screening inflammatory PM and poor pregnancy outcomes in tropical countries.

Conclusions

In summary, these data suggest that the peripheral IL-10 level may be useful as a biomarker of inflammation due to PM. Future studies should measure antenatal levels of

IL-10, and assess its relationship to parasitemia and pregnancy outcomes, and its utility for monitoring interventional trials. The sensitivity and specificity of peripheral IL-10 levels at delivery suggest that they may not be sufficient to be used clinically as diagnostic tools. Additional biomarkers of PM, placental inflammation and PM-related poor outcomes are needed to improve the clinical management of this major public health problem.

Authors' contributions

T.K.M., M.F., and P.E.D. designed and managed the MOMS Project. E.R.K. and J.D.K. performed the multiplex cytokine assay. A.M. performed PCR and histology studies. A.M. analyzed the data and wrote the manuscript with assistance from other authors.

Conflict of interest: The authors declare no competing financial interests

Acknowledgements

The authors gratefully acknowledge the participation of the mothers and their infants in the MOMS Project, and the work of the MOMS Project staff, including assistant medical officers, nurses, village health workers, laboratory technicians, microscopists, and data entry personnel. Gretchen Langdon of Institute of International Health, Brown University, organized the cytokine assays.

This work was supported by grants from Bill & Melinda Gates Foundation (grant 29202), NIH (R01 AI52059 and TW05509) and Puget Sound Partners for Global Health to P.E.D.

References

1. Ismail MR, Ordi J, Menendez C, Ventura PJ, Aponte JJ, Kahigwa E, Hirt R, Cardesa A, Alonso PL: **Placental pathology in malaria: a histological, immunohistochemical, and quantitative study**. *Hum Pathol 2000*, **31**:85-93.
2. Fried M, Duffy PE: **Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta**. *Science* 1996, **272**:1502-1504.
3. Bulmer JN, Rasheed FN, Francis N, Morrison L, Greenwood BM: **Placental malaria. I. Pathological classification**. *Histopathology* 1993, **22**:211-218.
4. Duffy PE: **Immunity to malaria during pregnancy: different host, different parasite**. In: *Malaria in Pregnancy: deadly parasite, susceptible host: 2001; London, New York*: Taylor & Francis; 2001: 71-126.
5. Fried M, Muga RO, Misore AO, Duffy PE: **Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes**. *J Immunol* 1998, **160**:2523-2530.
6. Mockenhaupt FP, Ulmen U, von Gaertner C, Bedu-Addo G, Bienzle U: **Diagnosis of placental malaria**. *J Clin Microbiol 2002*, **40**:306-308.
7. Mockenhaupt FP, Bedu-Addo G, von Gaertner C, Boye R, Fricke K, Hannibal I, Karakaya F, Schaller M, Ulmen U, Acquah PA Dietz E, Eggelte TA, Bienzle U: **Detection and clinical manifestation of placental malaria in southern Ghana**. *Malaria J* 2006, **5**:119.
8. Ostrowski SR, Shulman CE, Peshu N, Staalsoe T, Hoyer-Hansen G, Pedersen BK, Marsh K, Ullum H: **Elevated plasma urokinase receptor predicts low birth weight in maternal malaria**. *Parasite Immunol 2007*, **29**:37-46.

9. Coutinho HM, McGarvey ST, Acosta LP, Manalo DL, Langdon GC, Leenstra T, Kanzaria HK, Solomon J, Wu H, Olveda RM, Kurtis JD, Friedman JF: **Nutritional status and serum cytokine profiles in children, adolescents, and young adults with Schistosoma japonicum-associated hepatic fibrosis, in Leyte, Philippines**. *J Infect Dis* 2005, **192**:528-536.

10. Muehlenbachs A, Fried M, Lachowitzer J, Mutabingwa TK, Duffy PE: **Genome-wide expression analysis of placental malaria reveals features of lymphoid neogenesis during chronic infection**. *J Immunol* 2007, **179**:557-565.

11. [http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html]

12. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE: **Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.** *J Exp Med* 1991, **174**:1209-1220.

13. Gazzinelli RT, Oswald IP, James SL, Sher A: **IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages**. *J Immunol* 1992, **148**:1792-1796.

14. Silva JS, Morrissey PJ, Grabstein KH, Mohler KM, Anderson D, Reed SG: **Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection**. *J Exp Med 1992*, **175**:169-174.

15. Heinzel FP, Sadick MD, Mutha SS, Locksley RM: **Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis.** *Proc Natl Acad Sci USA* 1991, **88**:7011-7015.

16. Sher A, Fiorentino D, Caspar P, Pearce E, Mosmann T: **Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection**. *J Immunol* 1991, **147**:2713-2716.

17. Bogdan C, Vodovotz Y, Nathan C: **Macrophage deactivation by interleukin 10**. *J Exp Med 1991*, **174**(6):1549-1555.

18. Rockett KA, Awburn MM, Cowden WB, Clark IA: **Killing of Plasmodium falciparum in vitro by nitric oxide derivatives**. *Infect Immun* 1991, **59**:3280-3283.

19. Wenisch C, Parschalk B, Narzt E, Looareesuwan S, Graninger W: **Elevated serum levels of IL-10 and IFN-gamma in patients with acute Plasmodium falciparum malaria**. *Clin Immunol Immunopathol* 1995, **74**:115-117.

20. Gosi P, Khusmith S, Looareesuwan S, Sitachamroom U, Glanarongran R, Buchachart K, Walsh DS: **Complicated malaria is associated with differential elevations in serum levels of interleukins 10, 12, and 15**. *Southeast Asian J Trop Med Public Health* 1999, **30**:412-417.

21. Suguitan AL, Jr., Leke RG, Fouda G, Zhou A, Thuita L, Metenou S, Fogako J, Megnekou R, Taylor DW: **Changes in the levels of chemokines and cytokines in the placentas of women with Plasmodium falciparum malaria**. *J Infect Dis* 2003, **188**:1074-1082.

22. Kurtzhals JA, Adabayeri V, Goka BQ, Akanmori BD, Oliver-Commey JO, Nkrumah FK, Behr C, Hviid L: **Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria**. *Lancet* 1998, **351**:1768-1772.

23. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V: **A low interleukin-10 tumor necrosis factor-alpha ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya.** *J Infect Dis* 1999, **179**:279-282.

24. Hugosson E, Montgomery SM, Premji Z, Troye-Blomberg M, Bjorkman A: **Higher IL-10 levels are associated with less effective clearance of Plasmodium falciparum parasites.** *Parasite Immunol* 2004, **26**:111-117.

25. Fried M, Domingo GJ, Gowda CD, Mutabingwa TK, Duffy PE: **Plasmodium falciparum: chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta.** *Exp Parasitol* 2006, **113**:36-42.

26. Urban BC, Cordery D, Shafi MJ, Bull PC, Newbold CI, Williams TN, Marsh K: **The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria.** *Infect Immun* 2006, **74**:6700-6706.

27. Rogerson SJ, Brown HC, Pollina E, Abrams ET, Tadesse E, Lema VM, Molyneux ME: **Placental tumor necrosis factor alpha but not gamma interferon is associated with placental malaria and low birth weight in Malawian women.** *Infect Immun* 2003, **71**:267-270.

28. Das BS, Thurnham DI, Das DB: **Influence of malaria on markers of iron status in children: implications for interpreting iron status in malaria-endemic communities.** *Br J Nutr* 1997, **78**:751-760.

29. Taylor PG, Martinez-Torres C, Mendez-Castellano H, Bosch V, Leets I, Tropper E, Layrisse M: **The relationship between iron deficiency and anemia in Venezuelan children**. *Am J Clin Nutr* 1993, **58**:215-218.

30. Rogers JT, Bridges KR, Durmowicz GP, Glass J, Auron PE, Munro HN: **Translational control during the acute phase response. Ferritin synthesis in response to interleukin-1**. *J Biol Chem* 1990, **265**:14572-14578.

Figure legends

Figure 1. Receiver operator curve for peripheral IL-10 levels in first time mothers to detect PM. Solid line is the best fit curve; dashed lines show the 95% confidence intervals.

Figure 2. Peripheral IL-10 levels stratified for maternal parity, PM and the presence of inflammatory cells by placental histology. P-value was calculated using Mann-Whitney test. P0, primigravidae; P1+, multigravidae.

Figure 3. Relationship of peripheral IL-10 levels and placental TNF-α mRNA levels in first time mothers. Gene expression is presented as 2^x fold expression over KRT7. Simple regression analysis was used to calculate R and P-values.

Table 1: Characteristics of the study population. *

	Primigravidae			Multigravidae		
Characteristic	**PM- (n = 166)**	**PM+ (n = 39)**	**P**	**PM- (n = 415)**	**PM+ (n = 40)**	**P**
Maternal age in years (Mean; SD)	20.6 (3.3)	19.7 (1.9)	0.0964	28.9 (5.9)	25.7 (4.4)	**0.0008**
Birth weight in kg (Mean; SD)	3.10 (0.43)	2.83 (0.42)	**0.001**	3.25 (0.386)	3.04 (0.36)	**0.0014**

**Data presented are Mean (SD).*

Table 2: Peripheral levels of cytokines and other soluble factors stratified by parity and PM status.*

	Primigravidae			Multigravidae		
Factor	**PM- (n = 166)**	**PM+ (n = 39)**	**P**	**PM- (n = 415)**	**PM+ (n = 40)**	**P**
TNF	22.9 [8.3-46.7]	62.1 [26.3-127.5]	<0.0001	18.7 [8.70-37.9]	57.7 [23.8-84.7]	0.0002
TNF- RI	948 [550-1411]	1374 [851-2290]	0.0003	812 [481-1249]	1004 [517-1580]	0.0978
TNF- RII	186 [0-494]	673 [260-1425]	<0.0001	190 [0-403]	590 [129-959]	<0.0001
IL-1	2.28 [0.72-4.36]	5.05 [1.70-11.3]	0.0018	2.06 [0.73-4.46]	3.06 [0.32-7.36]	0.1409
IL-4	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.7676	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.9054
IL-5	1.96 [0.39-4.21]	1.76 [0.12-3.32]	0.4215	2.09 [0.39-4.09]	1.90 [0.89-5.89]	0.3158
IL-6	18.4 [9.26-37.6]	29.3 [15.0-42.5]	0.083	13.1 [3.75-29.5]	13.2 [8.83-19.2]	0.7618
IL-10	5.69 [3.28-11.3]	23.4 [15.1-62.7]	<0.0001	6.12 [2.91-11.6]	22.2 [13.9-40.3]	<0.0001
IFN-γ	0.0 [0.0-0.0]	0.0 [0.0-3.67]	0.5083	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.6607
Leptin	2404 [973-6218]	1029 [658-4115]	0.0245	2161 [1014-5293]	1490 [452-5052]	0.3444
Ferritin	14.0 [8.00-32.4]	62.7 [20.8-144.8]	<0.0001	11.6 [6.7-25.9]	40.4 [19.3-82.0]	<0.0001

**Data are presented as median [interquartile ranges]. P-values were calculated by Mann-Whitney test.*

Table 3: Area under the Receiver Operator Characteristic (ROC) curve to detect PM.*

Soluble factor	All gravidities	Primigravidae
TNF	0.690	0.731
TNF- RI	0.635	0.694
TNF- RII	0.731	0.752
IL-1	0.608	0.658
IL-10	0.815	0.830
Ferritin	0.733	0.759

**Only cytokines and other soluble factors significantly elevated during PM are shown.*

Table 4: Sensitivity and specificity of discrete IL-10 cut-off levels to classify cases of PM in first time mothers (n=205).

IL -10 levels	Sensitivity (%)	Specificity (%)
≥ 10 pg/mL	84.6	72.9
≥ 15 pg/mL	79.5	84.3
≥ 35 pg/mL	43.6	95.8

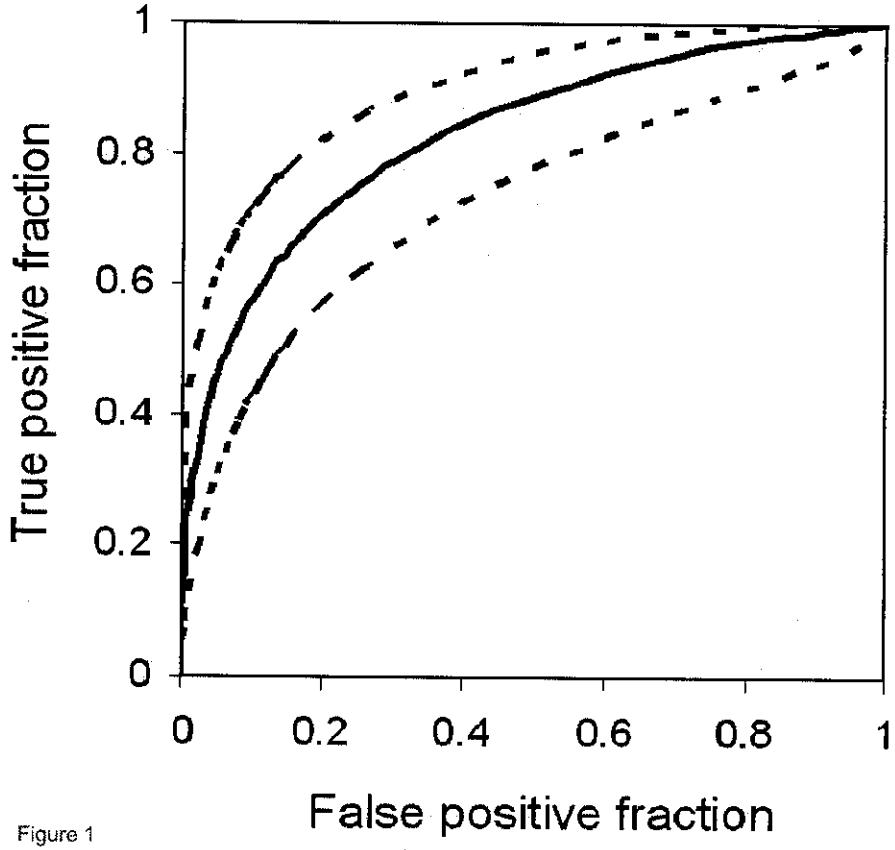
True positive fraction
False positive fraction
0
0.2
0.4
0.6
0.8
1

Figure 1

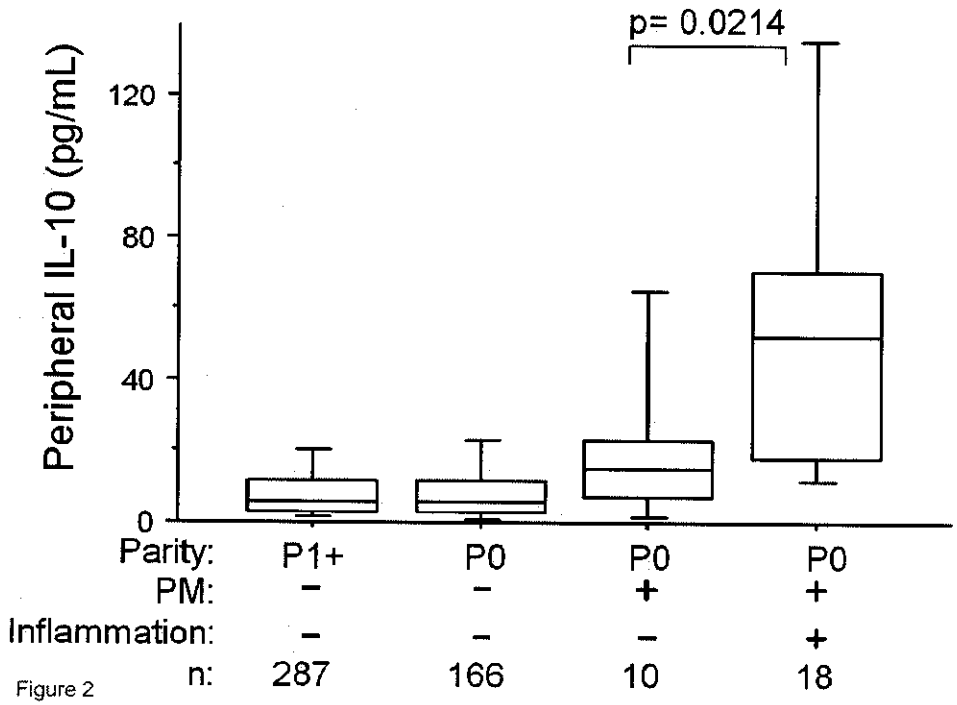
p= 0.0214
Peripheral IL-10 (pg/mL)
120
80
40
0
Parity: P1+ P0 P0 P0
PM: − − + +
Inflammation: − − − +
n: 287 166 10 18

Figure 2

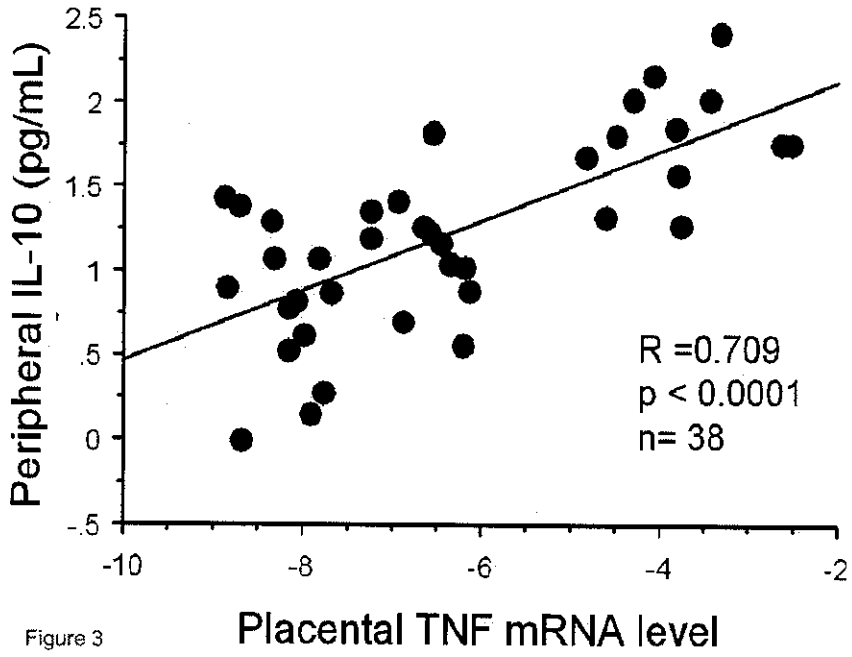
Peripheral IL-10 (pg/mL)
2.5
2
1.5
1
.5
0
-.5
-10
-8
-6
-4
-2
R =0.709
p < 0.0001
n= 38
Placental TNF mRNA level

Figure 3